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Contents

THE PREPARATION OF FLEXIBLE COLLODION MEMBRANES.

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(Received for publication, November 10, 1921.)

Collodion membranes were first utilized for experiments in diffusion by Fick (1) in 1855. Fick realized their advantages but was unable to develop a suitable method of fastening them to holders and so abandoned their use.

Since his paper was published many authors have described various methods of making and applying these membranes of which a fairly complete bibliography may be found in the paper of Bigelow and Gemberling (2).

The numerous attempts which have been made to control the permeability of these membranes have not been attended by any marked success. It was early known that these membranes became more impermeable on drying, and this fact was utilized by Bigelow and Gemberling (2), and Malfitano (3) to control the permeability of their membranes. No attempt was made to standardize the membranes made in this way as to the degree of permeability except to state that the longer the membranes were allowed to dry the less permeable they became. A more serious attempt was made in this direction by Walpole (4), who controlled his membranes by measuring the thickness of the film with a micrometer. He allowed a definite weight of collodion to evaporate on a glass plate of known area to given thickness and thus obtained a definite degree of drying, which could be accurately described by giving the ratio of the weight of the film to its weight when completely dry.

Brown (5) describes a method in which the permeability is varied by treating the dry membranes with different strengths of ethyl alcohol. He dips a small test-tube into an 8 per cent solution of collodion in a mixture of equal parts of absolute ethyl

alcohol and ether, and allows the membrane to drain for 5 minutes while held in a vertical position in an inverted Erlenmeyer flask. The tube is immediately immersed in water for about 1 minute, and the hardened collodion film is then stripped off. The membrane thus obtained is allowed to dry over night at room temperature, which renders it highly impermeable. When these membranes are soaked for 24 hours in different strengths of alcohol their permeability increases correspondingly to the increase in the concentration of the alcohol. The most permeable membranes were obtained by treatment with 97 per cent alcohol, but these membranes were extremely fragile and very apt to rupture.

All the membranes described by these various authors have a common disadvantage in that they become brittle and stiff when permitted to become completely dry and are then rendered impermeable and very easily broken. This necessitates the keeping of these membranes under water. The membranes described by the writer in this paper are extremely flexible even after being allowed to dry for a period of 2 weeks at room temperature and still retain their permeability. Some of these membranes have been rolled up in a ball like a wad of tissue paper without being injured in the slightest as is shown by the fact that when blown out and filled with a saturated solution of ammonium chloride they exhibited a high degree of endosmosis. A difference in level of 25 cm. in a tube 5 mm. in diameter has been observed when such a membrane has been placed in distilled water for 5 minutes.

This remarkable flexibility in the dried collodion membranes is obtained by adding ethyl acetate to solutions of collodion in mixtures of absolute alcohol and dry ether. The method of preparation is as follows.

5 gm. of "Anthony's Negative Cotton," which has been dried for 48 hours over concentrated sulfuric acid, are placed in a clean and dry Erlenmeyer flask and 25 cc. of absolute ethyl alcohol are added, and the flask is agitated so that all the cotton is thoroughly moistened. 75 cc. of ether, which has been distilled from sodium, are now added and the flask is shaken until the cotton has completely dissolved. 15 cc. of ethyl acetate are now added with shaking to secure complete mixing of the solvents. The solution is allowed to stand over night and then the clear supernatant liquid is decanted off into another flask.

After preparing numerous solutions of varying amounts of solvents, the proportions of solvents given above have been found to give the best results. It has been found that increasing the amount of ethyl acetate at first increases the flexibility of the resulting membrane rapidly, but that the effect diminishes as the concentration is increased so that the maximum flexibility is reached at a concentration of about 40 per cent. It has also been found that increasing the concentration of the ethyl acetate tends to diminish the permeability of the membrane to a slight extent. Acetone also seems to decrease the permeability of the membrane in proportion to its concentration.

The membranes are prepared inside of test-tubes or Kjeldahl flasks and may be made of any desired size, but the smaller ones are more easily obtained free from defects and of a more uniform thickness. The solution is poured into the flask, which must be perfectly clean and dry, and the excess of collodion is allowed to drain back into the container by holding the flask at an angle of about 60° and slowly rotating it until the collodion no longer drips freely and then the flask is clamped upside down in a stand and left until it is completely dry. Drying may be hastened by blowing a gentle blast of air into the flask, or even better by inserting a tube, connected with a slight suction, into the flask, taking care not to touch the side of the vessel. When the membrane is perfectly dry it is removed by peeling the top of the film from the neck of the flask and then pouring a gentle stream of water between the membrane and the side of the flask. This frees the membrane and it can then be withdrawn. This method differs from those previously described in that the membrane is allowed to become perfectly dry before being placed in contact with water, while in previous methods the membrane must be immersed in water before it dries or it becomes impermeable.

The chief difficulty encountered by the writer was the appearance of pinholes in the membranes especially in those made in the larger flasks. These pinholes can be avoided by taking care that the flasks in which the membranes are cast are scrupulously clean and dry, and that the collodion solutions are allowed to settle until all the particles of dust and undissolved matter have collected at the bottom of the container and are then carefully decanted into a dust-free vessel. The membranes can be made more uniform in thickness by following the technique of Farmer (6).

The membranes are tested for pinholes and other defects by filling them with a strong solution of Congo red and placing them in a beaker of distilled water. Any leakage is easily detected by the appearance of the dye in the beaker. The permeability of the membranes is established by the readiness with which they allow potassium ferrocyanide to dialyze out from a normal solution into distilled water. No attempt has been made to determine the permeability of the membranes except as to whether they permit Congo red to pass or not. It is interesting to note, however, in this regard that a solution of Bence-Jones protein after being dialyzed against distilled water until free from salt gave no test for the protein in the original solution.

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STUDIES ON EXPERIMENTAL RICKETS.

XII. IS THERE A SUBSTANCE OTHER THAN FAT-SOLUBLE A ASSOCIATED WITH CERTAIN FATS WHICH PLAYS AN IMPORTANT RÔLE IN BONE DEVELOPMENT?

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Plate 1.

(Received for publication, November 1, 1921.)

Mellanby (1) was the first to associate a protective function of certain fats with bone development. He observed that certain diets caused changes in the epiphyses of the long bones in dogs which appeared, insofar as could be detected through radiography, to be rachitic in nature. The inclusion of butter fat or cod liver oil led to the development of bones in which radiographs showed no abnormalities. He was thus led to postulate the theory that the dietary essential, fat-soluble A, or some substance which has a similar distribution in nature exerted an antirachitic effect.

In an earlier paper, we presented some evidence that there is in cod liver oil a substance capable of exerting a favorable influence on bone growth which is distinct from fat-soluble A (antixerophthalmic substance (2)). We have pursued our investigations with a view to determining whether it is the fat-soluble A or some other substance which protects the skeleton when the calcium content of the diet is unfavorable to the formation of normal bone. In the present paper we present a series of records of groups of rats which were fed throughout the period of growth and over a considerable part of adult life on diets which were closely

comparable except that on one hand certain groups received butter fat, and on the other certain groups received cod liver oil. The defect in these diets, aside from the lack of or subminimal provision of a hypothetical bone-nourishing substance, was limited to deficiency of calcium. The phosphorus content was in every case not far from the optimal.

Under such regulated conditions of nutrition where the lack of calcium is very pronounced and the amount of phosphorus is adequate, the provision of a liberal amount of some organic substance which is associated with certain fats exerts a most remarkable effect in improving the well being of the animals. The problem we discuss in this paper is whether this substance is furnished by both butter fat and cod liver oil, and whether but one or more than one substance is involved in the enhancement of diets low in fat-soluble A when either of these fats is added to such diets.

The line of evidence presented in the charts is as follows: On a diet such as we employed, young rats are much better nourished, when supplied with 1 per cent of cod liver oil than with 10 to 20 per cent of butter fat, as is shown by better growth, fertility, success in rearing young, and in length of life. This is true, notwithstanding, that 3 per cent of butter fat is ample for providing the animals with sufficient fat-soluble A and any other organic substance exerting a special effect on the bones, when the content of calcium in the diet is raised to approximately half the optimal. The provision of nearly seven times this amount does not exert much protection to the animals against the specific detrimental effect of lack of calcium when the content of the diet in this element is from one-fifteenth to one-fifth or sixth the optimal amount. 1 per cent of cod liver oil, on the other hand, seems to increase in a very remarkable manner the effectiveness with which the anatomic elements of the body tissues deal with a very low calcium supply. Our results indicate that there is no progressive benefit to the animals brought about by the exhibition of greater and greater amounts of butter fat. This would indicate that the effects are not proportional to the amount of this fat which is included in the diet. Furthermore, it appears that 1 to 2 per cent of cod liver oil supplies as much of the substance which exerts a peculiar influence on bone

growth as the animals can profit by even when the calcium supply is very low indeed. An extensive experience in feeding diets of the type here described has failed to reveal any evidence that a rat is benefited by the inclusion of more than 5 per cent of butter fat when the calcium and phosphorus are normal.

It is further shown from the experimental data recorded in the charts that as the calcium content of the diet is increased the differences between the effects of butter fat and cod liver oil tend to disappear, and vanish completely or nearly so when the content of calcium reaches as much as one-half or more of the optimal amount.

The results of this series of experiments were so consistent and decisive that we can deduce no other conclusion than that cod liver oil contains in abundance some substance which is present in butter fat in but very slight amounts, and which exerts a directive influence on the bone development and enables animals to develop with an inadequate supply of calcium much better than they could otherwise do. This substance is apparently distinct from fat-soluble A, which is essential for growth and which is associated definitely with the prevention of ophthalmia (keratomalacia).

We are continuing these investigations along other lines which are yielding results which bear directly on the confirmation or refutation of these conclusions.

Hart and Steenbock have recently published results which harmonize with the view we have expressed above. They found that the administration of butter fat did not influence favorably the calcium balance in a goat, whereas under the dietary conditions employed by them the feeding of cod liver oil promptly changed the calcium balance from negative to positive (3).

In presenting the experimental data contained in this paper we have expressed the belief that it tends strongly to support the view that there are two distinct organic factors operating in the nutrition of a mammal which are associated with certain fats. One serves especially to fortify the defensive mechanism of the body against the effects of lack of calcium. We are, however, already in possession of very definite data of entirely different kinds from those discussed above, which indicate clearly that similar effects can be induced in animals deprived of calcium by

illumination with sunlight as well as by the action of cod liver oil (4). This observation, together with an appreciation of the fact that much work of a very refined nature must still be carried out before we fully understand the problems of growth of the osseous tissues, leads us to point out at this time our belief that the formation of definite conclusions either from the work of others or from our own would best be deferred until other experimental work now developing is completed.

Chart 1. Lots 2925 and 2934 were fed on diets which were essentially alike except that one contained 10 per cent and the other 20 per cent of butter fat. Both diets contained not far from the optimal content of phosphorus, but contained only about one-fifteenth the optimal amount of calcium (about 0.400 gm. of phosphorus and 0.050 gm. of calcium). Other factors in the diets were satisfactorily constituted. The significant point which we wish to emphasize in connection with these records is that the animals grew very poorly and had very short lives, as contrasted with animals fed a similar mixture containing 0.5 per cent of calcium carbonate and but 3 per cent of butter fat (Chart 16). This shows that the limiting factor in the growth of these animals was the calcium supply. This demonstrates that all factors other than calcium are so constituted as to fully meet the nutritive needs of the growing rat when sufficient calcium is available. Although some animals in Lot 2925 lived some weeks longer than the average in Lot 2934, we regard this as of no significance in connection with differences in butter fat content. Animals on diets of this type are in a condition of nutritional instability and the length of life of any individual as compared with another will depend on its natural vitality. When compared with Lots 2732 and 2733 (Chart 2) these records show that 10 to 20 per cent of butter fat fails to protect the growing rat against the effects of lack of sufficient calcium in the same manner as does 1 to 3 per cent of cod liver oil.

Chart 2. Lots 2732 and 2733 were fed diets essentially comparable in their properties to those described in Chart 1, except that the former received 1 and 3 per cent respectively of cod liver oil instead of 10 to 20 per cent of butter fat. Both diets contained essentially the same content of phosphorus and of calcium (0.390 gm. of phosphorus and 0.050 gm. of calcium per 100 gm. of food), as did those of Lots 2925 and 2934.

It is a very remarkable thing that the animals in these two groups grew much better than those in Chart 1. They were fairly fertile. Lots 2925 and 2934 were sterile. Lots 2732 and 2733 were fairly successful in rearing their young to the weaning age, soon after which age the young died. These animals presented a fairly well nourished appearance, whereas Lots 2925 and 2934, which received high intakes of butter fat, were very poorly developed and were of abnormal form (stocky), dirty and rough coated.

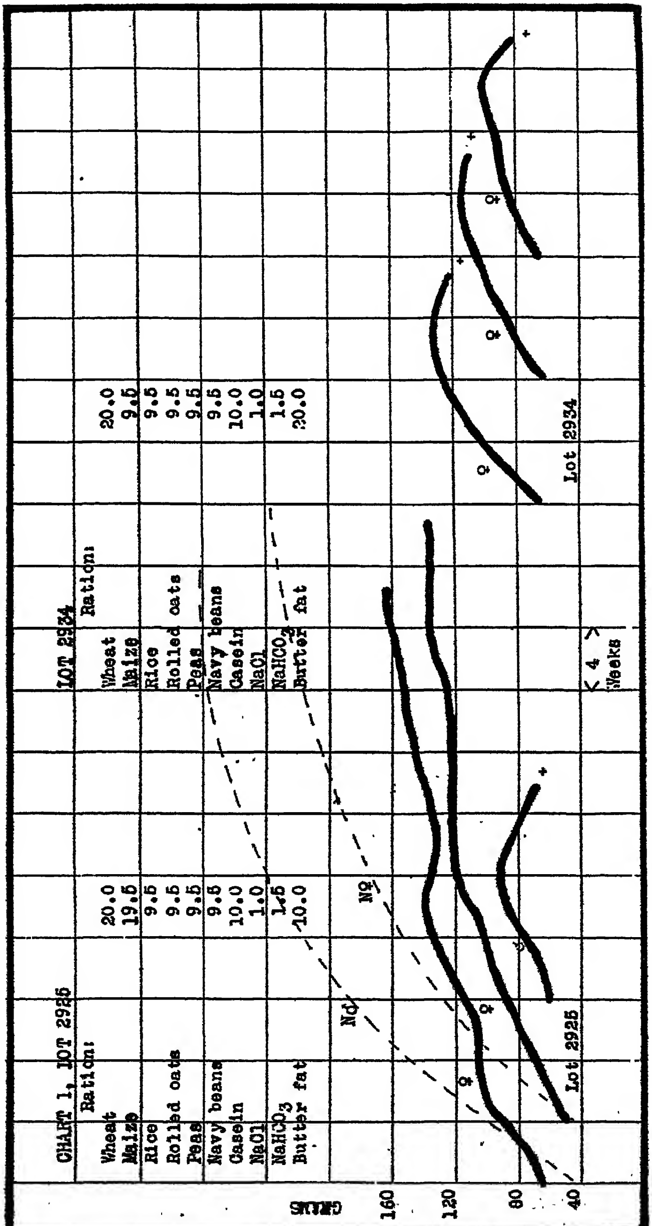
CHART 1, LOT 2925
Ration:
Wheat 20.0
Maize 19.5
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
Butter fat 10.0
LOT 2934
Ration:
Wheat 20.0
Maize 9.5
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
Butter fat 20.0
N♂
N♀
Lot 2925
Lot 2934
< 4 >
Weeks
GRAMS
160
120
80
40

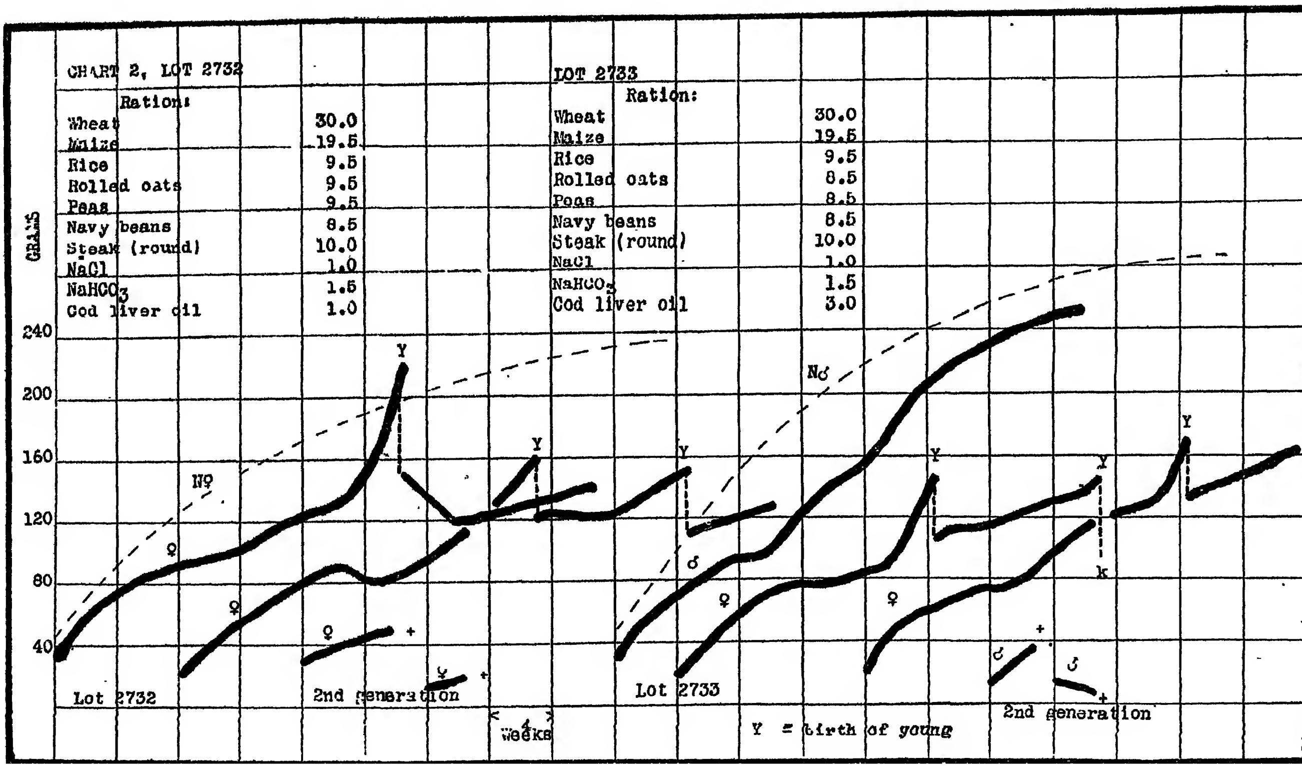

CHART 2, LOT 2732
Ration:
Wheat 30.0
Maize 19.5
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 8.5
Steak (round) 10.0
NaCl 1.0
NaHCO3 1.5
Cod liver oil 1.0
LOT 2733
Ration:
Wheat 30.0
Maize 19.5
Rice 9.5
Rolled oats 8.5
Peas 8.5
Navy beans 8.5
Steak (round) 10.0
NaCl 1.0
NaHCO3 1.5
Cod liver oil 3.0
GRAMS
240
200
160
120
80
40
N♀
N♂
Y
♀
♂
+
k
Lot 2732
2nd generation
4 weeks
Lot 2733
Y = birth of young
2nd generation

Cod liver oil, we have repeatedly observed with different diets, protects growing rats against the injurious effects of lack of calcium and enables them to grow and appear well nourished for a considerable period, where they would fail to grow and would be very inferior with even much greater amounts of butter fat instead of the cod liver oil.

It is especially remarkable that whereas 3 per cent of butter fat suffices when added to food mixtures of the type under discussion to meet all the requirements of the rat for fat-soluble A and for the specific calcium-depositing factor, if there be one, when the diet contains favorable concentrations of calcium, 10 to 20 per cent of butter fat fails to shield them in a manner at all comparable with 1 per cent of cod liver oil. Again, it is remarkable that animals specifically fasted for calcium are protected as effectively by 1 per cent of cod liver oil as by 3 per cent or higher planes of intake.

We do not believe that our samples of butter fat could differ in the concentration of the calcium-depositing substance, since, as will be shown in later charts, when the diet contains calcium in amounts ranging from 0.1 to 0.5 per cent of the carbonate, butter fat becomes adequate to supply the nutritive needs of the body and the differences in the effects of cod liver oil and of butter fat in the diet tend to disappear.

Chart 3. Lot 2821 was fed a diet essentially comparable to those discussed in the preceding charts except that its content of 20 per cent of casein raised the phosphorus content of the food mixture. Like the preceding diets it was very poor in calcium. 5 per cent of butter fat did not suffice to protect the animals against lack of calcium. This chart should be compared with Chart 4, Lot 2822, which differed significantly only in containing 2 per cent of cod liver oil and 3 per cent of butter fat.

Lot 2821 was very poorly nourished and became badly deformed. The coats of these rats were rough and thin (see Fig. 1) and they aged very early.

Chart 4. Lot 2822 had a diet exactly similar to that of Lot 2821 (Chart 3) except that 2 per cent of cod liver oil replaced 2 per cent of butter fat. This modification of the diet made a remarkable difference in their growth and well being (see Fig. 2). The former were stunted, infertile, and short lived. The latter grew to full normal size, presented a well nourished appearance, and were fairly fertile, and succeeded in rearing a considerable number of their young to the weaning age. The young were puny, pot-bellied, almost completely stunted in growth, and died early. The mothers declined rapidly after nursing two or three litters. The males, while well nourished for an interval following the completion of growth, soon presented a poorly nourished appearance and aged early.

The effect of the cod liver oil was to make the animals in some degree immune for a time to the injurious effects of lack of calcium. Even ten times as much butter fat could not do this. If a small addition of calcium were made to this diet the butter fat would supply sufficient of some organic

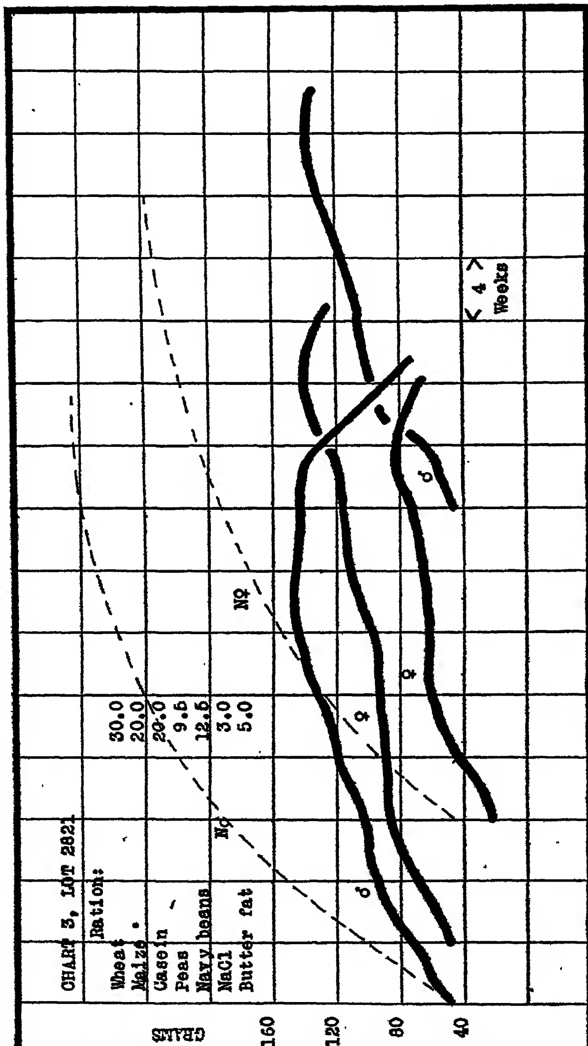
CHART 3, LOT 2821
Ration:
Wheat 30.0
Maize 20.0
Casein 20.0
Peas 9.5
Navy beans 12.5
NaCl 3.0
Butter fat 5.0
GRAMS
160
120
80
40
4 Weeks
N♀
♂
♀

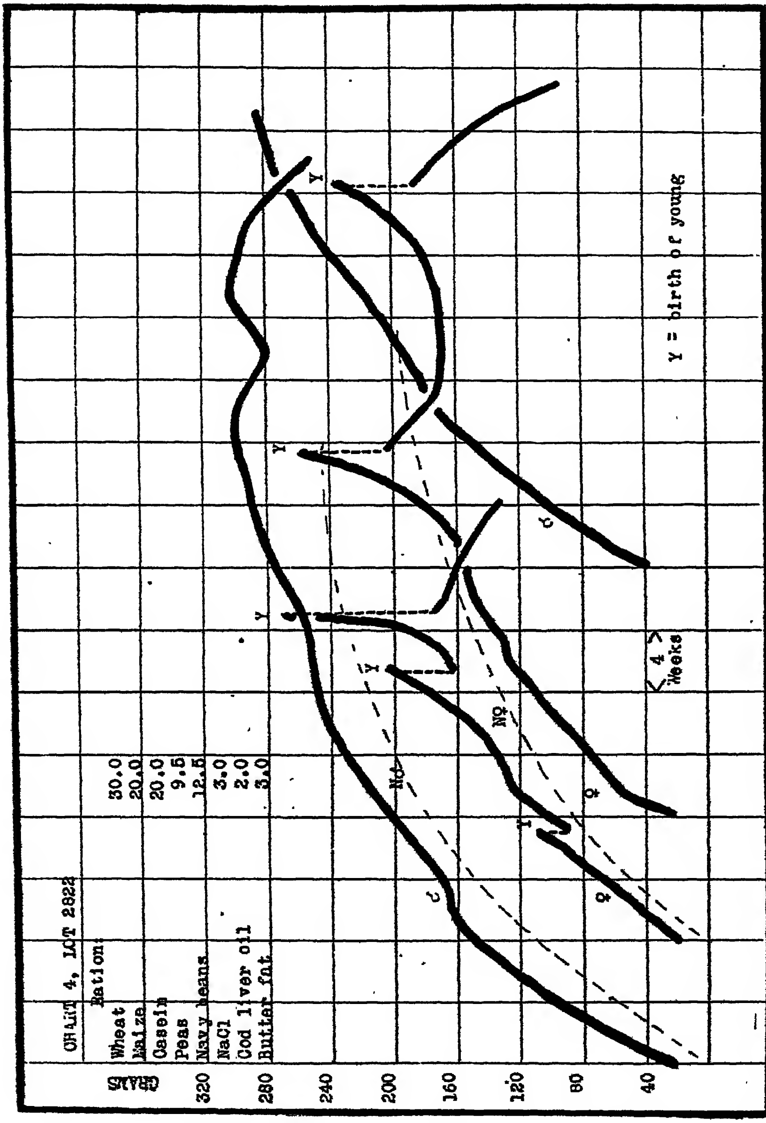
CHART 4, LOT 2822
Ration:
Wheat 30.0
Maize 20.0
Casein 20.0
Peas 9.5
Navy beans 12.5
NaCl 3.0
Cod liver oil 2.0
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
Y = birth of young
4 Weeks

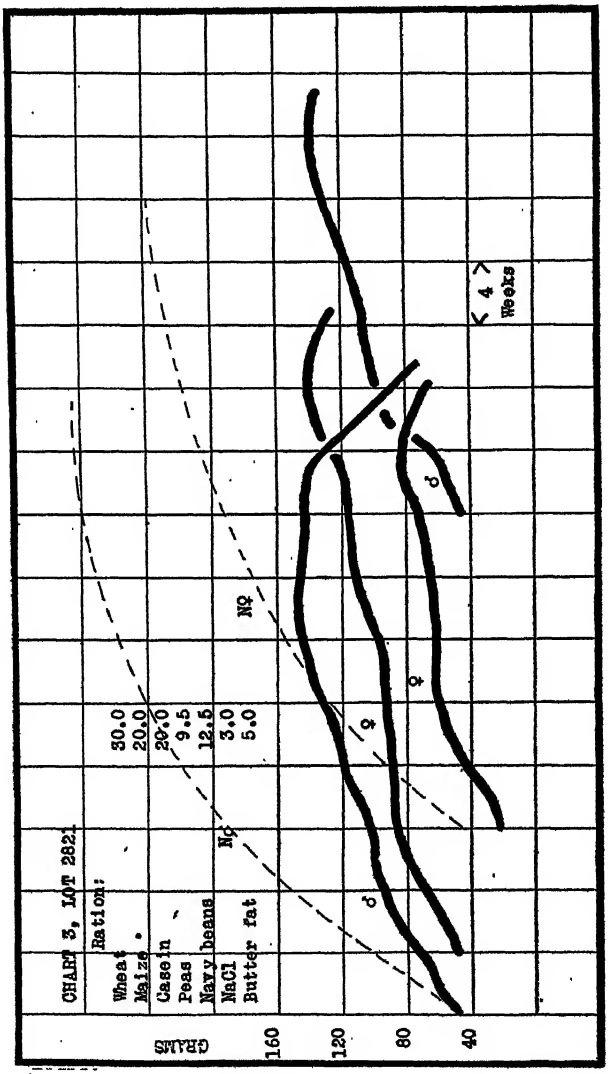
CHART 3, LOT 2821
Ration:
Wheat 30.0
Maize 20.0
Casein 20.0
Peas 9.5
Navy beans 12.5
NaCl 3.0
Butter fat 5.0
N♀
N♂
♀
♀
♂
♂
< 4 > Weeks
GRAMS
160
120
80
40

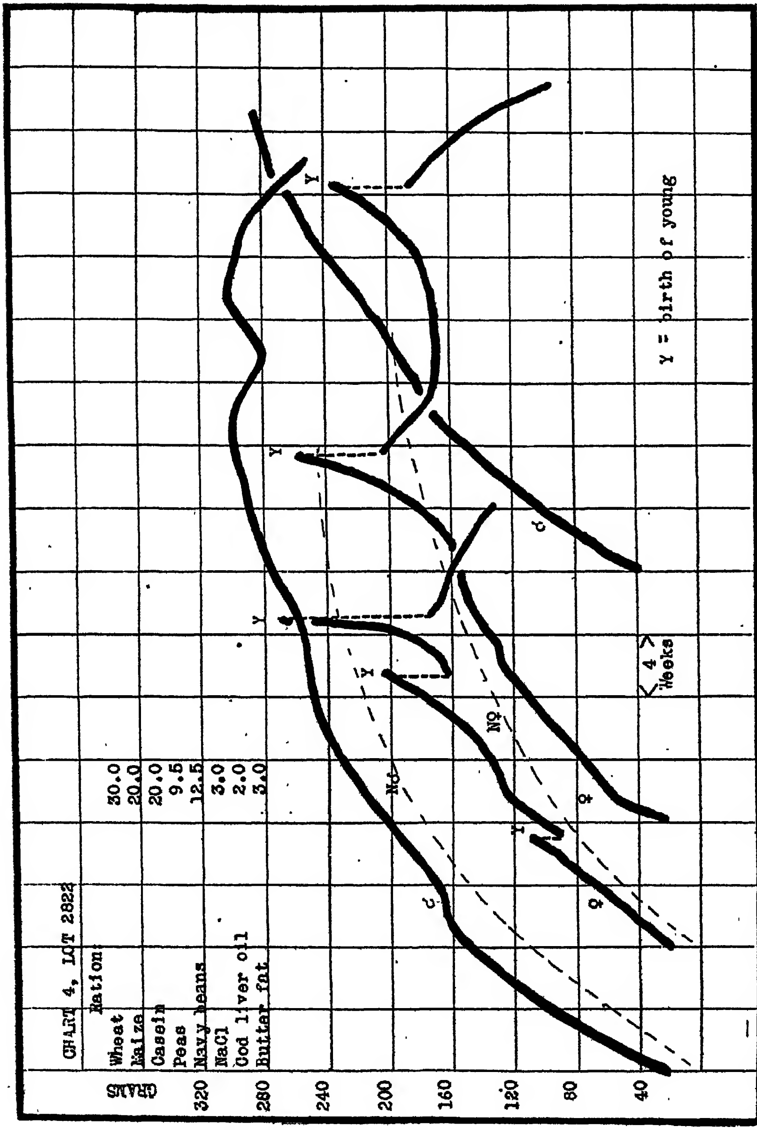
CHART 4, LOT 2822
Ration
Wheat 30.0
Maize 20.0
Casein 20.0
Peas 9.5
Navy beans 12.5
NaCl 3.0
Cod liver oil 2.0
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
Y = birth of young
4 Weeks
Y
♂
♀
N♂
N♀

substance intimately associated with the development of the osseous system, and the animals would develop normally. In order to differentiate between the nutritive value of cod liver oil and of butter fat *in a qualitative way* it must be administered with a diet poor in calcium, for with an adequate supply of calcium and phosphorus in the food either serves equally well to supplement such diets as are here discussed.

Chart 5. Lot 2766. This and succeeding groups of experimental animals illustrate the comparative value of cod liver oil and of butter fat in the presence of suboptimal amounts of calcium, where the phosphorus content of the diet is near the optimum. A small amount of calcium (0.045 gm.) was added in the 5 per cent of milk powder but this was too small to contribute much to the well being of the animals.

These records should be compared with those of Chart 6 (Lot 2765), whose diet was almost identical except that it contained 1 per cent of cod liver oil instead of 10 per cent of butter fat. The rats were protected in a remarkable way against the effects of lack of calcium by this small amount of cod liver oil. 10 per cent of butter fat with this diet afforded some protection, but although this amount is at least three times that required to meet all the needs of the rat for protection against ophthalmia and to enable it to develop a normal skeleton when the diet contains the optimal amount of calcium, it fails to supply enough of some substance intimately concerned with bone formation when the calcium intake is low. These results point to the existence of a specific calcium-depositing substance distinct from fat-soluble A (antixerophthalmic substance).

On this diet the animals grew slowly but never attained the full adult size. The females were capable of producing several litters each but the infant mortality was high and they early developed signs of senility. The second generation confined to this food supply was greatly stunted and inferior. Their forms were very short and stocky. They had large deposits of body fat.

Chart 6. Lot 2765 should be contrasted with Lot 2766 (Chart 5). The significant difference in the composition of the diets of these two groups was in the nature of the fats which they contained. Both diets were far below the optimal in their content of calcium, but were otherwise well constituted. 1 per cent of cod liver oil very effectively protected these animals against the harmful effects of calcium starvation because of its content of some organic substance which appears to be distinct from that substance (fat-soluble A) which is essential for growth and is a specific agent in preventing ophthalmia of dietary origin. The protection afforded by the cod liver oil is not complete. It consists in enabling growth to proceed and causes the animals to appear externally to be well nourished. This is very apparent when we contrast the animals described in Chart 5, which had the same diet with 10 per cent of butter fat, with those in Chart 6, which had 1 per cent of cod liver oil. This contrast we have repeatedly observed in rats fed other diets low in calcium where fat-soluble A was in one case supplied by cod liver oil and in the other by butter fat.

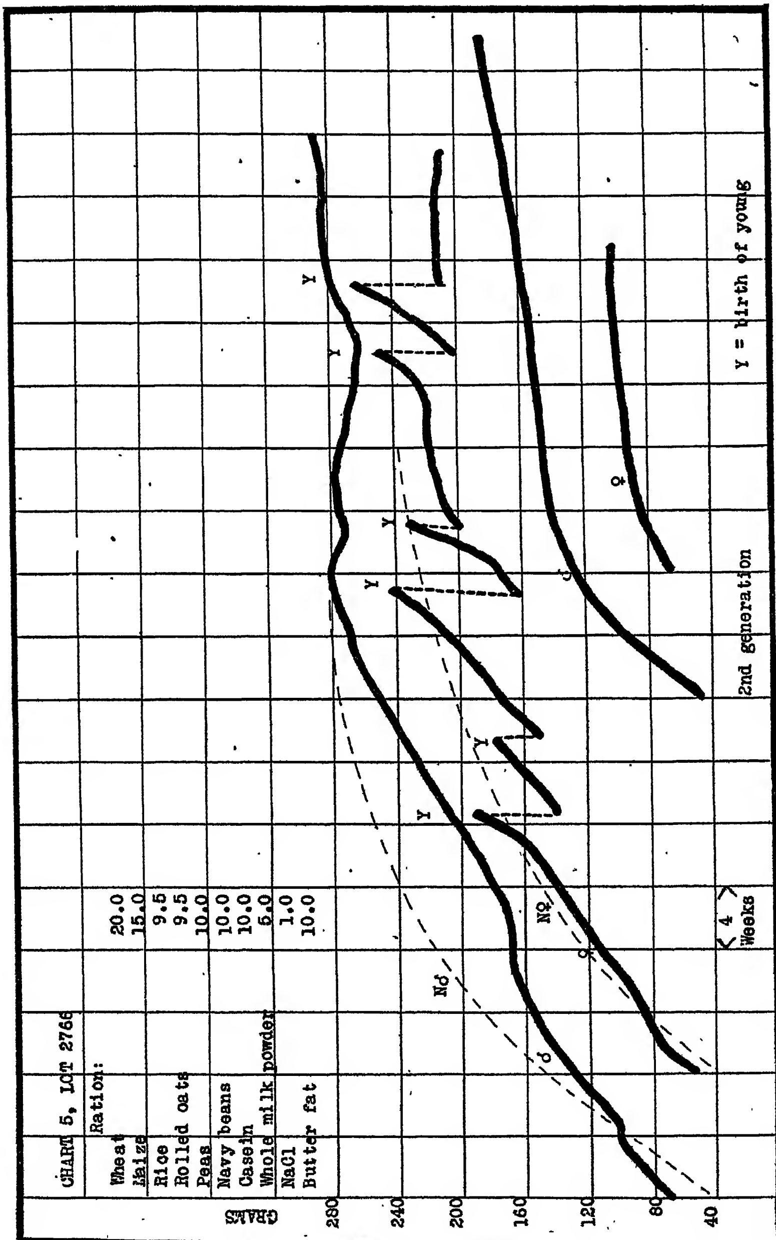
CHART 5, LOT 2766
Ration:
Wheat 20.0
Maize 15.0
Rice 9.5
Rolled oats 9.5
Peas 10.0
Navy beans 10.0
Casein 10.0
Whole milk powder 5.0
NaCl 1.0
Butter fat 10.0
GRAMS
280
240
200
160
120
80
40
Y = birth of young
2nd generation
4 Weeks
N♂
N♀
♂
♀
Y

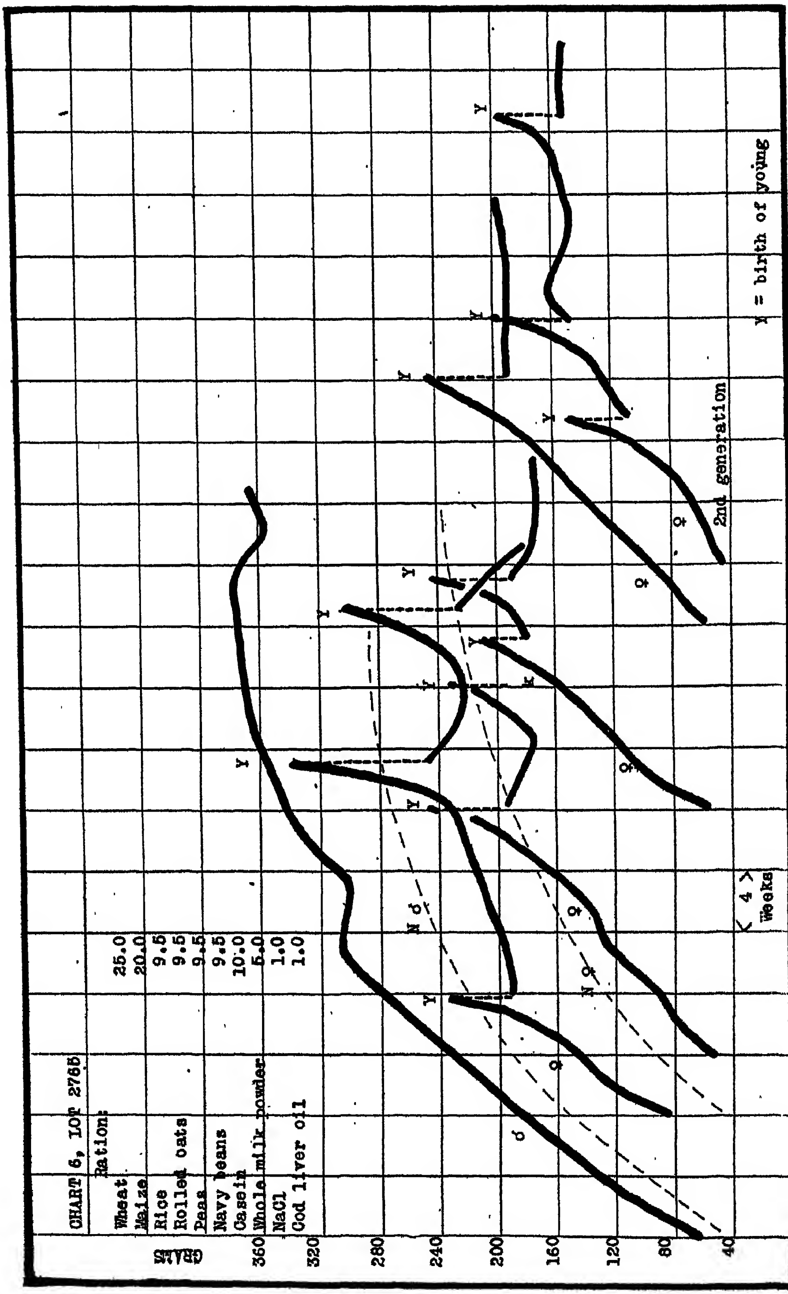
CHART 6, LOT 2765
Ration:
Wheat 25.0
Maize 20.0
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
Whole milk powder 5.0
NaCl 1.0
Cod liver oil 1.0
GRAMS
40
80
120
160
200
240
280
320
360
4 Weeks
N ♂
N ♀
♂
♀
Y
2nd generation
Y = birth of young

That the animals receiving cod liver oil were in a state of nutritional instability notwithstanding their good external appearance and fertility, is shown by the tendency of the females to collapse and die while nursing a second or third litter of young. Their skeletons were very poorly calcified and when the animals were boiled in water it was impossible to separate even the bones of the pelvis. The femur remained intact but was frequently deformed in second or third generation animals. The skull bones disintegrated in this treatment.

Succeeding generations tend to fail on this diet, but not as rapidly as rats on similar diets containing butter fat even in large amounts (ten to twenty times as much fat as in the cod liver oil diets). The small amount of calcium added in the 5 per cent of milk powder exerted an observable effect in improving the well being of these rats. This is easily seen by comparing Lots 2732 and 2733 (Chart 2) with Lot 2765 (Chart 6).

Chart 7. Lots 2957 and 2958. These groups, and those described in later charts, illustrate the beneficial effects of adding small amounts of calcium to the standard diet employed in most of the experiments here described. A comparison of Chart 1 with Chart 7 shows how marked is the effect in promoting the vitality of rats by the addition of even 0.1 to 0.2 per cent of calcium carbonate to the diet. Lot 2957 failed to grow as well with 0.1 per cent as did Lot 2958 with 0.2 per cent addition. The contrast is much greater in the vigor and capacity to grow of the young produced by these two groups. 0.2 per cent of calcium carbonate made it possible for the second generation to develop fairly well and produce young, whereas the addition of but 0.1 per cent did not enable the young appreciably to grow or to extend their lives beyond about 60 days after weaning. Both groups aged early and had abnormal forms. They were short and stocky. Even 1 per cent of cod liver oil makes rats on this diet develop long, lithe forms, although they fail early.

Charts 8 and 9. Lots 2952 (Chart 8) and 2953 (Chart 9) should be compared with Chart 7. The diets were the same except that in Chart 7 the fat addition consisted of 8 per cent of butter fat, whereas in Charts 8 and 9 it was 2 per cent of cod liver oil. Lot 2952 had 0.1 and Lot 2953 had 0.2 per cent of calcium carbonate added. The great superiority of cod liver oil over five to ten times as much butter fat is easily seen.

The general appearance of these animals was superior to the animals fed butter fat. Fertility was high and the mortality was low, although the nursing period was frequently prolonged beyond the normal time. The young did not look sleek and well nourished while depending on the mother, but later when placed on the family diet greatly improved in appearance.

Charts 10 and 11. Lot 2959 (Chart 10) should be contrasted with Lot 2954 (Chart 11). These show the growth and fertility of rats fed the basal diet discussed in preceding charts to which 0.3 per cent of calcium carbonate was added. In the former, 8 per cent of butter fat was added, whereas 2 per cent of cod liver oil was added to the latter. Both groups grew well,

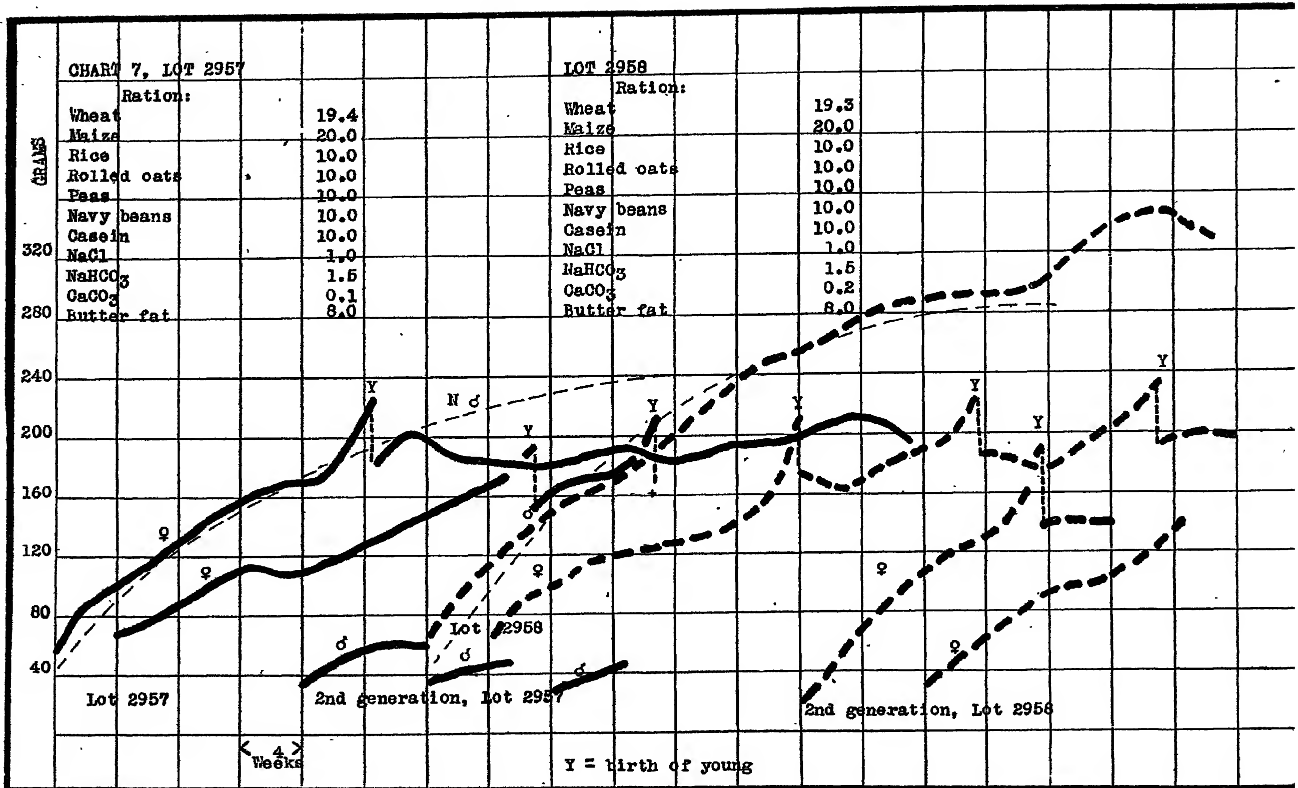
CHART 7, LOT 2957
Ration:
Wheat 19.4
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.1
Butter fat 8.0
LOT 2958
Ration:
Wheat 19.3
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.2
Butter fat 8.0
GRAMS
320
280
240
200
160
120
80
40
N ♂
Y
♀
♂
Lot 2958
Lot 2957
2nd generation, Lot 2957
2nd generation, Lot 2958
4 Weeks
Y = birth of young

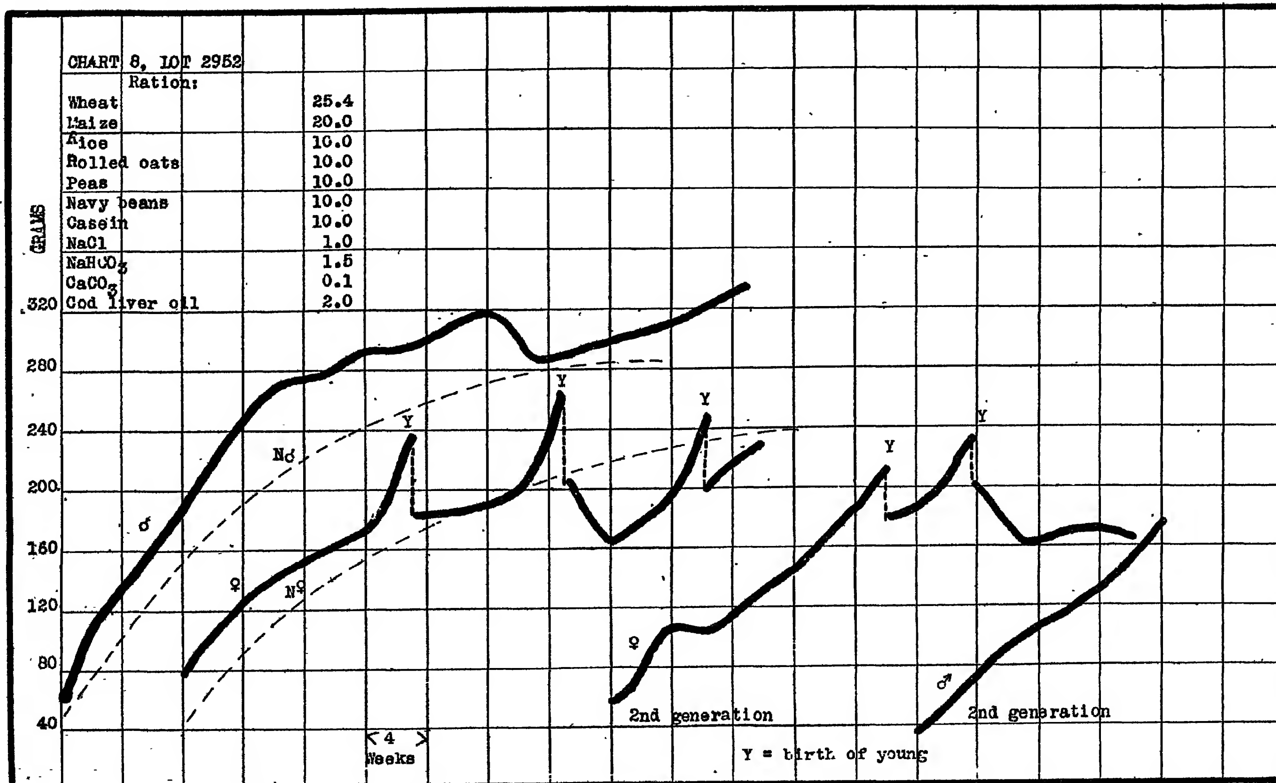
CHART 8, LOT 2952
Ration:
Wheat 25.4
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.1
Cod liver oil 2.0
GRAMS
320
280
240
200
160
120
80
40
♂
♀
N♂
N♀
Y
Y
Y
Y
Y
4 Weeks
♀
2nd generation
♂
2nd generation
Y = birth of young

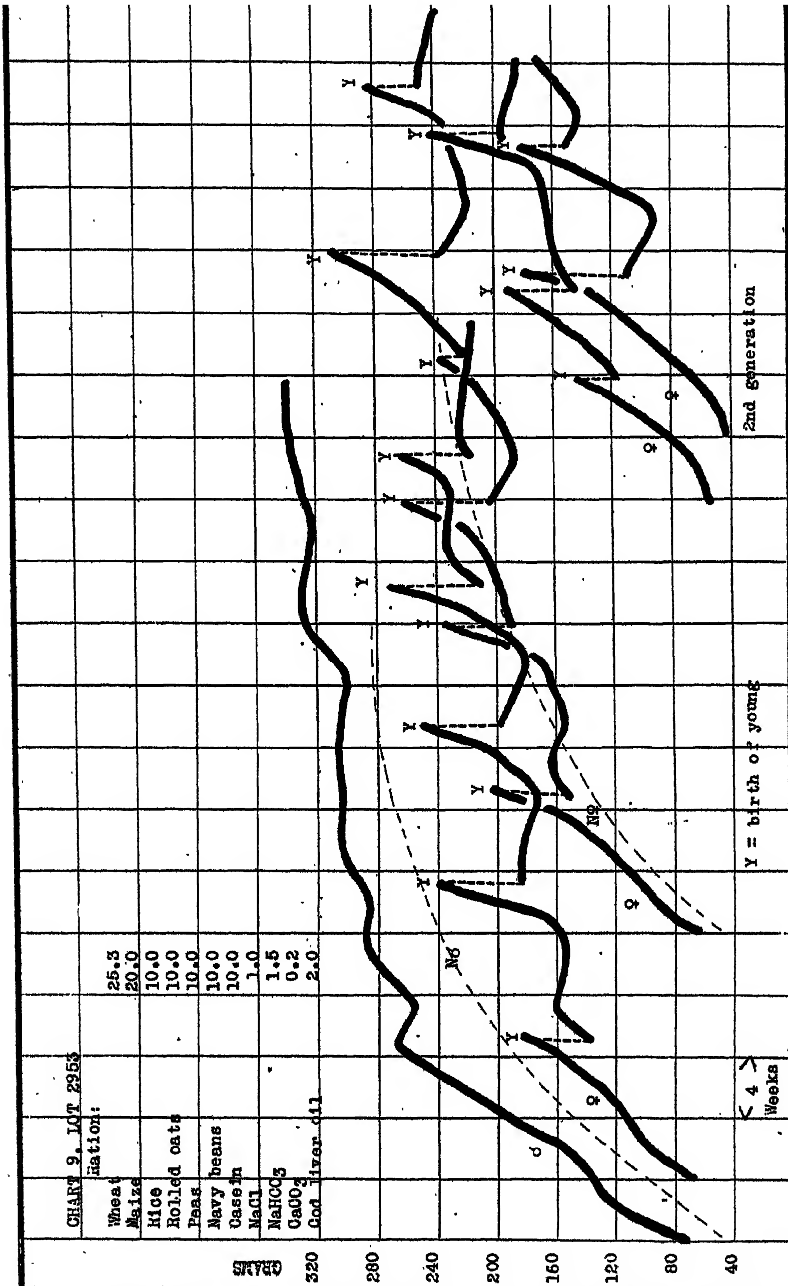
CHART 9. LOT 2953
Ration:
Wheat 25.3
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.2
Cod liver oil 2.0
GRAMS
320
280
240
200
160
120
80
40
Y = birth of young
2nd generation
< 4 >
Weeks

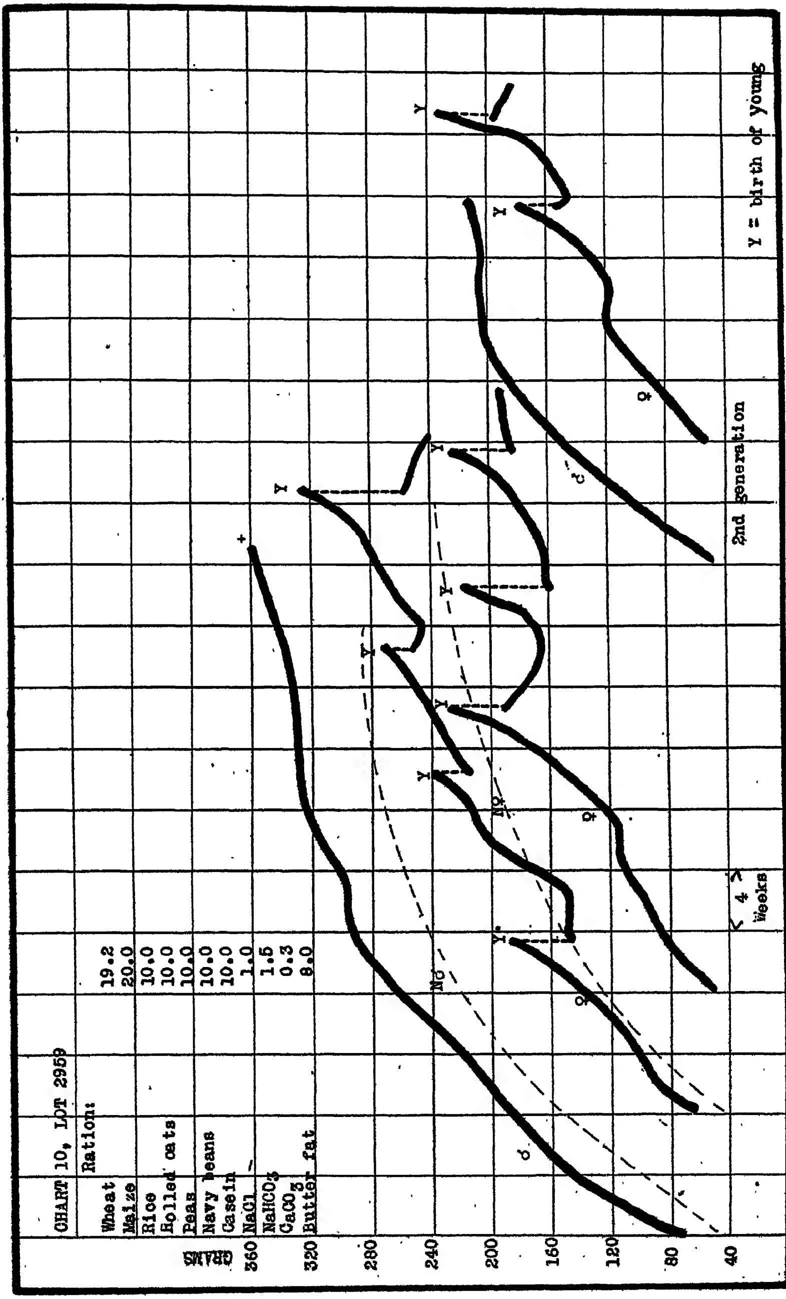
CHART 10, LOT 2959
Ration:
Wheat 19.2
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.3
Butter fat 8.0
GRAMS
360
320
280
240
200
160
120
80
40
Y = birth of young
2nd generation
4 Weeks
Y
♂
♀
Nc

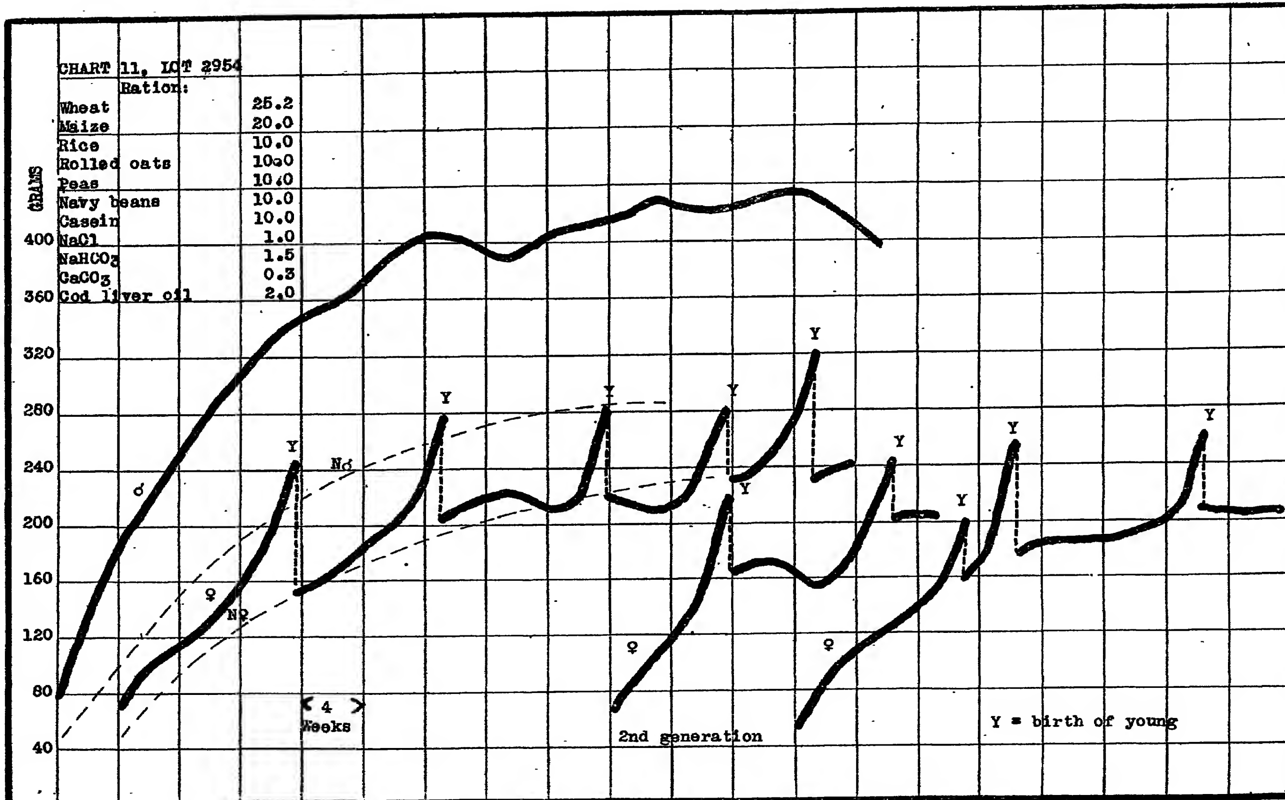
CHART 11, LOT 2954
Ration:
Wheat 25.2
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
NaHCO3 1.5
CaCO3 0.3
Cod liver oil 2.0
GRAMS
400
360
320
280
240
200
160
120
80
40
♂
♀
N♂
N♀
Y
4 Weeks
2nd generation
Y = birth of young

but it is easy to see that the group receiving the cod liver oil was superior to those getting butter fat. It is apparent from these and preceding charts that as higher additions of calcium are made to this otherwise satisfactory diet, the difference between the dietary qualities of butter fat and cod liver oil tend to disappear. This is further emphasized in Charts 12 to 15, in which higher planes of calcium intake were furnished.

Fertility was high in both these groups and rearing of the young was the rule. In these respects the cod liver oil group was more successful than the butter fat group. There was a corresponding difference in the smartness of their appearance.

Charts 12 and 13. Lots 2960 and 2955 correspond in every way to the two groups described in Charts 10 and 11, except that 0.4 per cent of calcium carbonate was added in each case. One diet contained 8 per cent of butter fat and the other 2 per cent of cod liver oil. The well being of these two groups presented less contrast than did those in Charts 10 and 11, but it was still possible to detect that those receiving cod liver oil were better nourished than those getting butter fat. This was shown especially in the behavior of the second generation, those from the cod liver oil achieving greater size and presenting a smarter appearance than those from the butter fat group. The animals described in Charts 10 to 16 inclusive showed essentially the same degree of solicitude in caring for their immature young.

Charts 14 and 15. Lots 2961 and 2956 continue the series now under discussion. They received 0.5 per cent of calcium carbonate in each case, and Lot 2961 had 8 per cent of butter fat, whereas Lot 2956 had 2 per cent of cod liver oil. With this moderate addition of calcium, which represents diets containing less than half the optimal content of this element, the differences in the supplementary value of butter fat and cod liver oil practically disappear. Both groups appeared to be nearly equally capable of growth and fertility and succeeded about equally well in rearing their young. The succeeding generations, including the third, showed but slight differences in vitality. There was, however, a slight advantage in favor of the cod liver oil.

Chart 16. Lot 2839 completes the argument we are presenting, to the effect that there are differences in the dietary properties of cod liver oil and of butter fat, which we can explain only on the assumption that we are dealing with two uncharacterized dietary factors in butter fat and cod liver oil. One of these is the antixerophthalmic substance, fat-soluble A. The other we suggest is a substance which plays a more important rôle in influencing the anatomic elements in the osseous system and may be designated as a calcium-depositing or phosphorus-mobilizing factor. Butter fat is richer in fat-soluble A than in the calcium-depositing factor. Cod liver oil is exceptionally rich in both substances.

It has only been found possible up to the present time to demonstrate the differences between these fats by using diets poor in calcium, for with

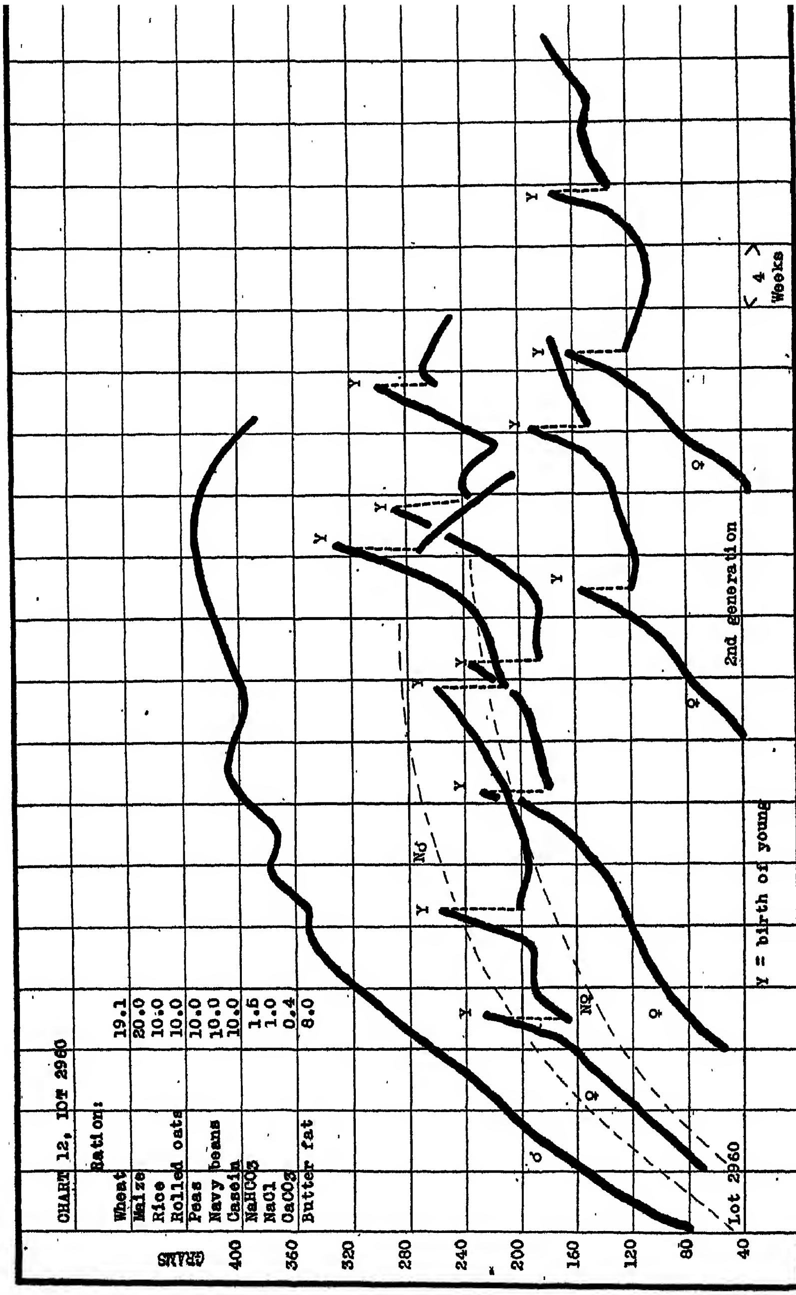
CHART 12, LOT 2960
Ration:
Wheat 19.1
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
$NaHCO_3$ 1.5
NaCl 1.0
$CaCO_3$ 0.4
Butter fat 8.0
GRAMS
400
360
320
280
240
200
160
120
80
40
Y = birth of young
2nd generation
Lot 2960
4 Weeks
N♂
N♀
♂
♀
Y

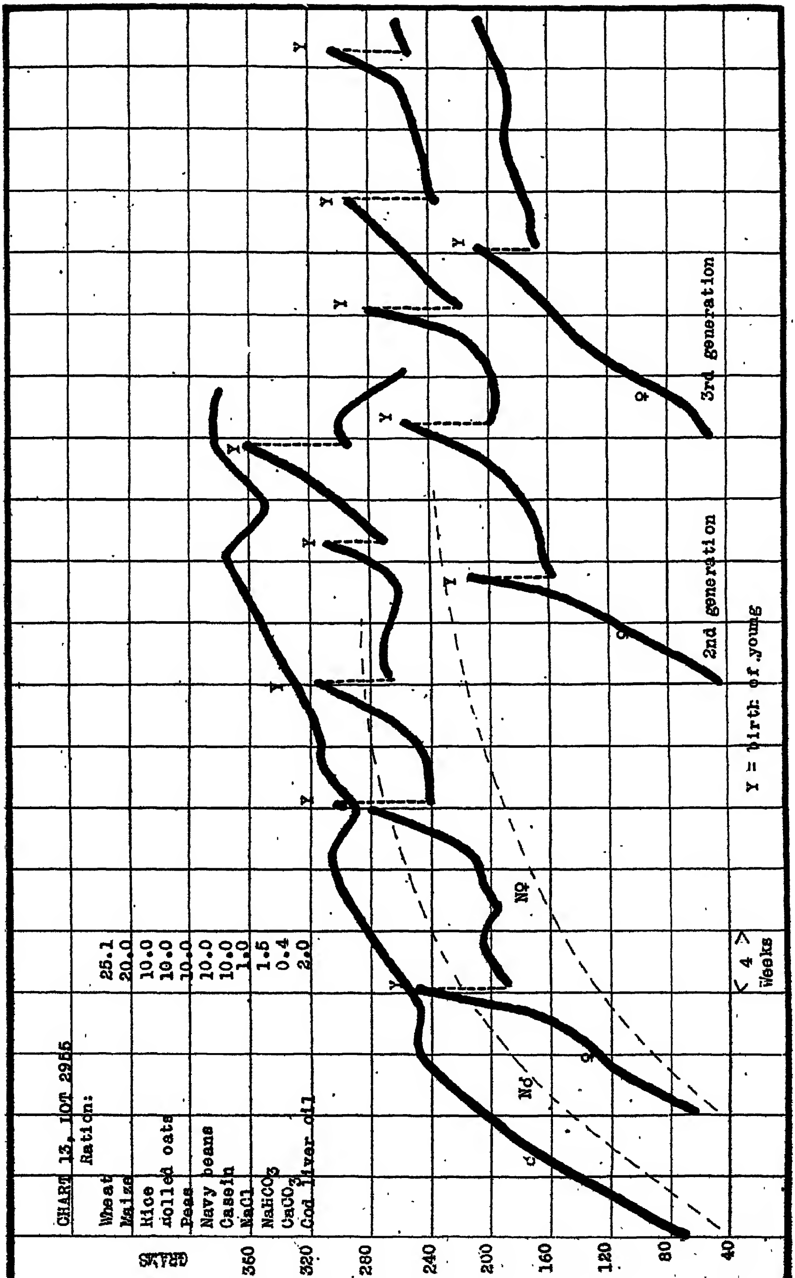
CHART 13, LOT 2955
Ration:
Wheat 25.1
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.4
Cod liver oil 2.0
GRAMS
360
320
280
240
200
160
120
80
40
N♂
N♀
♂
♀
Y
2nd generation
3rd generation
Y = birth of young
< 4 >
Weeks

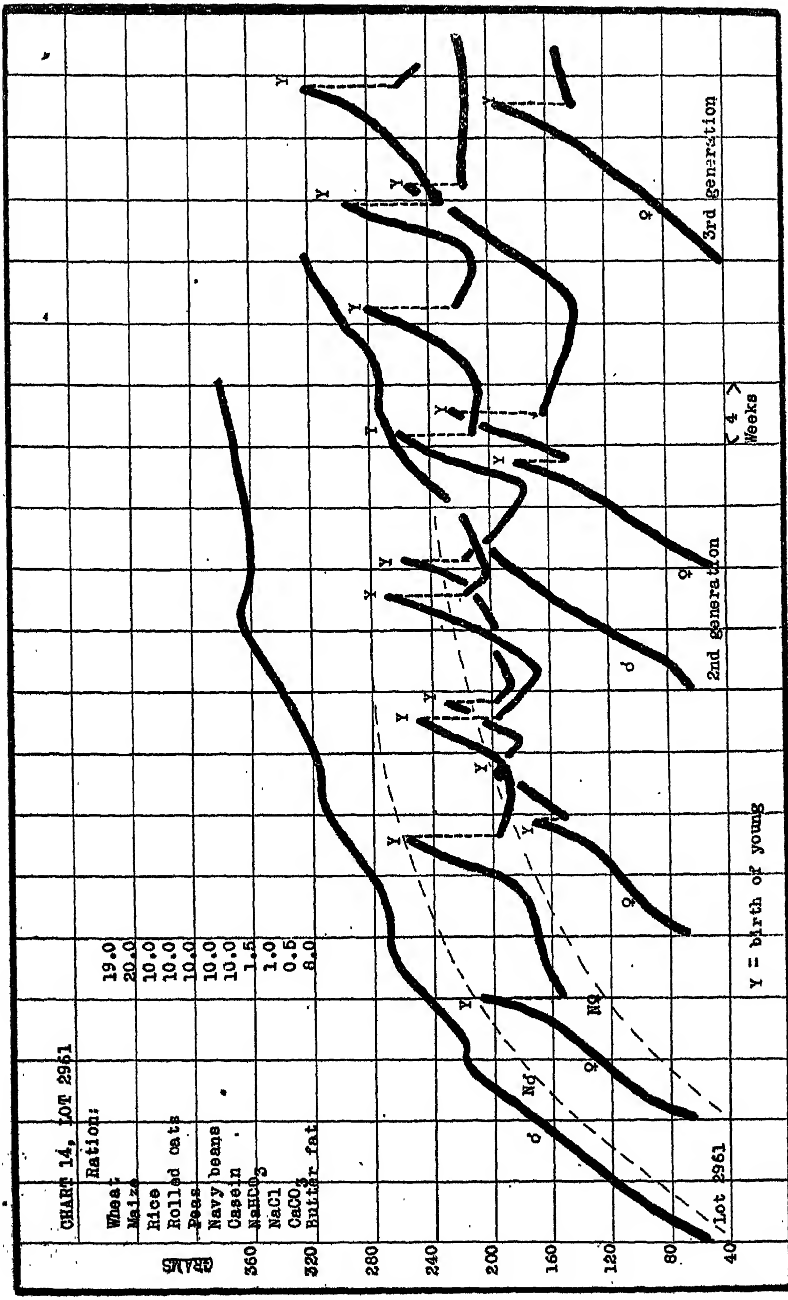
CHART 14, LOT 2961
Ration:
Wheat 19.0
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
$NaHCO_3$ 1.5
NaCl 1.0
$CaCO_3$ 0.5
Butter fat 8.0
GRAMS
360
320
280
240
200
160
120
80
40
Lot 2961
Y = birth of young
4 Weeks
2nd generation
3rd generation

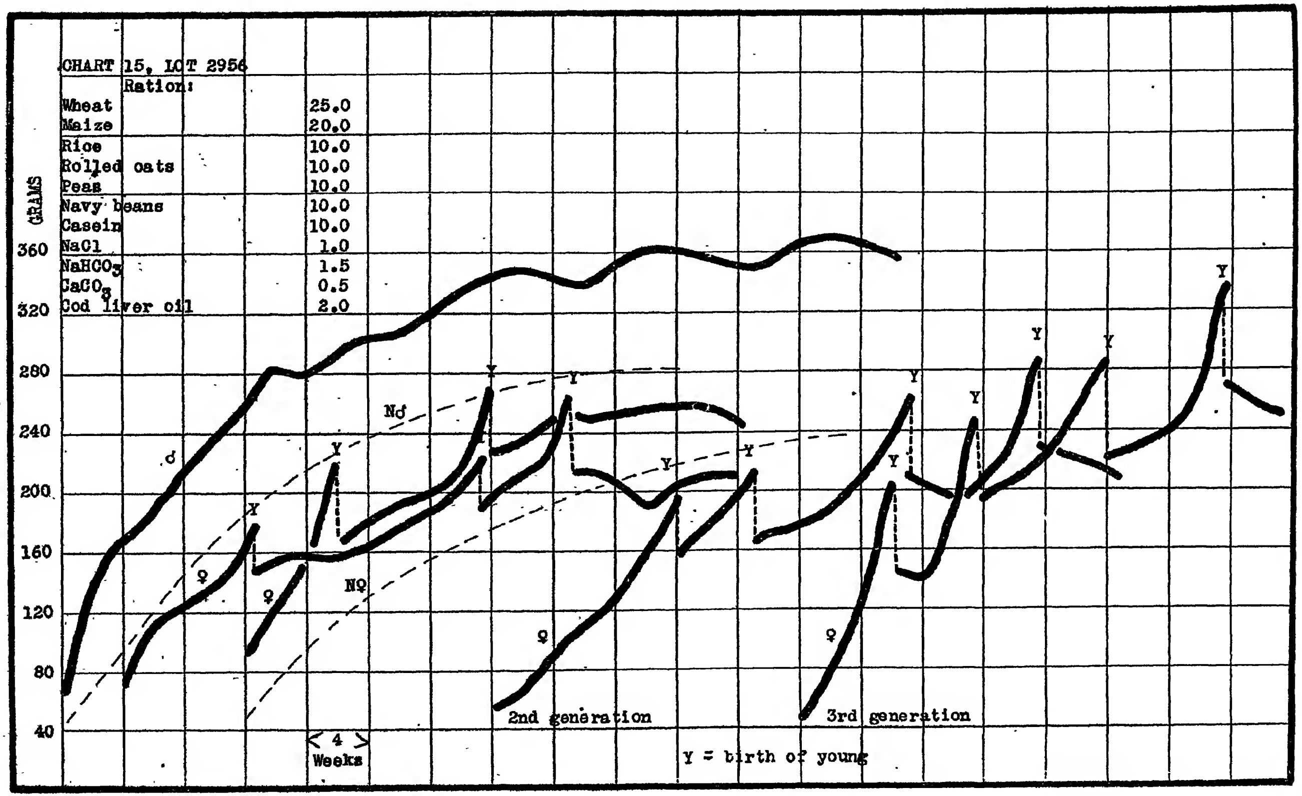
CHART 15, LOT 2956
Ration:
Wheat 25.0
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.5
Cod liver oil 2.0
GRAMS
360
320
280
240
200
160
120
80
40
♂
♀
♀
N♂
N♀
Y
2nd generation
3rd generation
< 4 > Weeks
Y = birth of young

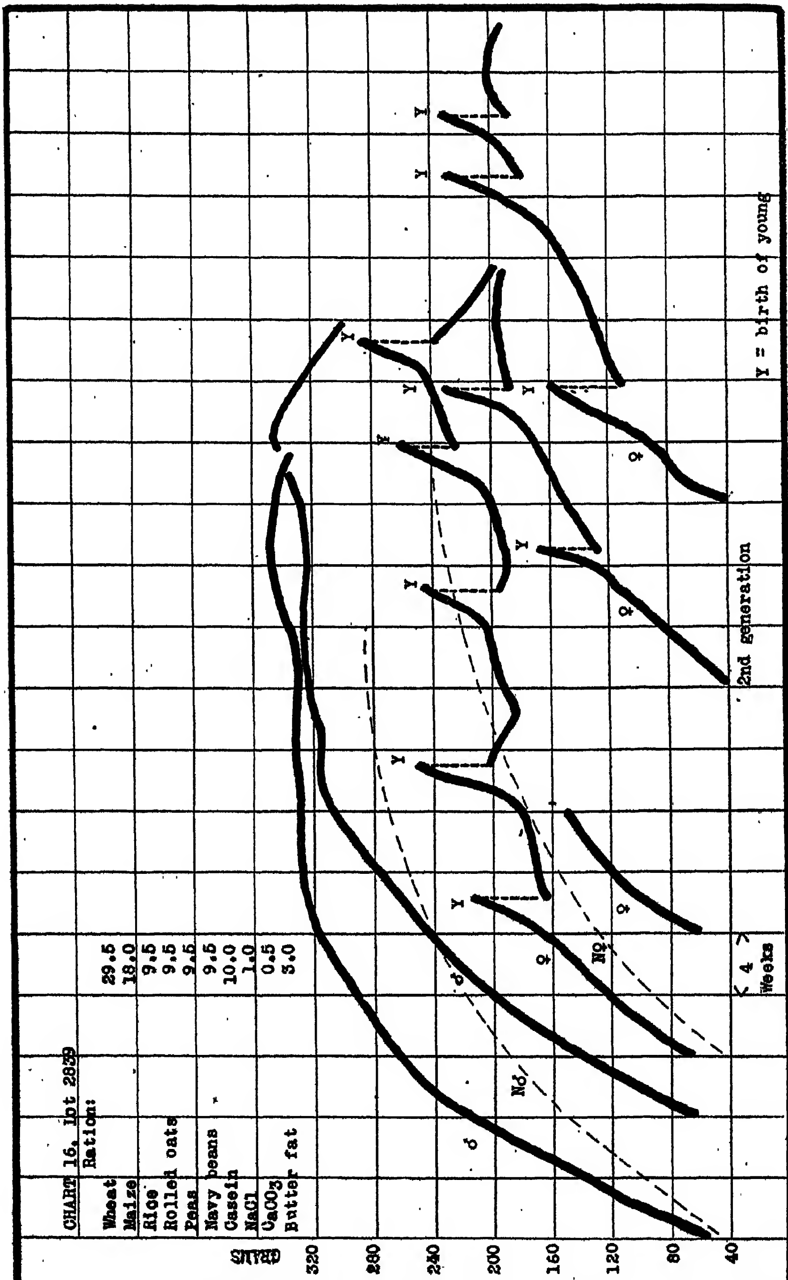
CHART 16. Lot 2839
Ration:
Wheat 29.5
Maize 18.0
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
NaCl 1.0
CaCO3 0.5
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
♂
N♂
♀
N♀
Y
2nd generation
Y = birth of young
4 Weeks

low intake of this element there is a correspondingly high requirement of the substance which we have spoken of as the calcium-depositing factor, but which is more than this. It exerts a distinct influence on the anatomic elements of the growing bone and enables the osteoblasts to form approximately the optimal amount of osteoid tissue. It leads to the deposition of calcium phosphate in a degree which is not possible in its absence when the calcium content of the diet is very low. It improves the general well being of the animals.

The most important point brought out by the records in this chart is that when 0.5 per cent of calcium carbonate was added to the diet, which still contained only about half the optimal content of calcium, even so small an amount of butter fat as 3 per cent sufficed to enable the animals to grow in a normal manner and to exhibit normal vitality as shown in fertility and success in rearing young. The second generation was somewhat undersized but was fertile.

This chart should be compared with Chart 1. Lot 2934 had nearly seven times the content of butter fat which was consumed by Lot 2839, yet in the absence of a calcium supply exceeding that furnished by a cereal and legume seed mixture they were not protected by this high butter fat intake.

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EXPLANATION OF PLATE 1.

FIG. 1 shows the appearance of a rat which was restricted to Diet 2821 (Chart 3). This diet was faulty in that its calcium content was too low and the phosphorus near the optimal. The fat in the diet consisted of 5 per cent of butter fat. Note the poor coat and general appearance of inferior development. This picture should be contrasted with Fig. 2, which had the same diet but with 2 per cent of cod liver oil and 3 per cent of butter fat.

FIG. 2. This rat from Lot 2822 was the same age as that shown in Fig. 1, and was confined to its experimenal diet for the same number of days (155). Its diet differed from that shown in Fig. 1 only in that 2 per cent of cod liver oil replaced 2 per cent of butter fat. Note the superior development, the good coat, and general appearance of physical well being brought about by the small amount of cod liver oil.

FIG. 1.

FIG. 2.

THE BIOGENESIS OF OIL OF PEPPERMINT.

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(*From the Wisconsin Pharmaceutical Experiment Station, Madison.*)

(Received for publication, October 25, 1921.)

It is commonly stated in the literature that the Japanese peppermint oil contains such a large proportion of menthol that it is a magma of crystals and saturated mother liquor even at ordinary temperature; that the European oils, on the other hand, have a minimum of free menthol; while the American oil is intermediate. The high menthol content of the Japanese oils has led to the introduction into Europe and America of strains of Japanese plants. The expectation that these would retain their chemical characteristics seems to have been justified in at least one instance.[1]

In studying the cohobated oils of American and Japanese peppermints grown by the Wisconsin Pharmaceutical Experiment Station, it was logically but a step further to expect that the Japanese mint oil would be exceedingly rich in menthol, and that the American mint oil would be composed of both menthol and menthone. This expectation was based on the fact that the oxygenated constituents of an oil are usually more soluble in water than the hydrocarbons, and hence are recovered from the aqueous distillate in relatively greater quantity by cohobation. Therefore, it was a distinct surprise to find that the 1920 cohobated oil of Japanese peppermint consisted almost wholly of pulegone;[2] and that the American mint oil, though having menthone[3] and menthol[4] as major constituents, contained methyl-1-cyclohexanone-3 as well.[5] The cohobated aqueous distillate

[1] See the Semiannual Report of Schimmel and Company, April, 1911, 92.

[2] Pulegone: Semicarbazone, M. P. 168° (recorded 167.5–168°).
Nitrosite, M. P. 83° (recorded 81.5°).

[3] Menthone: Semicarbazone, M. P. 185° (recorded 184°).

[4] Menthol: Pure crystals, M. P. 42.5°.

[5] Methyl-1-cyclohexanone-3: Semicarbazone, M. P. 180° (recorded 180°). The above experimental data were embodied in a paper presented to the New Orleans Meeting of the American Pharmaceutical Association, September, 1921.

was found to contain acetone.[6] The last two compounds are the products of hydrolysis of pulegone. Accordingly it was thought that both mints produced pulegone, but that it was subsequently hydrolyzed in the American mint by metabolic processes.

Further reflection on the problem and discussion with others emphasized the fact that in recent years the peppermints have been considered as hybrids rather than as true species or varieties. Whereupon the opportunity for the comparison of morphological with chemical hereditary characteristics seemed too good to pass by. At present, the American mint is considered to be a cross between *Mentha aquatica* Linné and *Mentha spicata* Hudson. The known chemical constituents of each oil and a possible scheme of their biogenesis is given below. The data are taken from Gildemeister.[7]

Mentha aquatica, Linné (*Mentha citrata*, Ehrhardt) × *Mentha spicata*, Hudson (*Mentha viridis*, Linné)

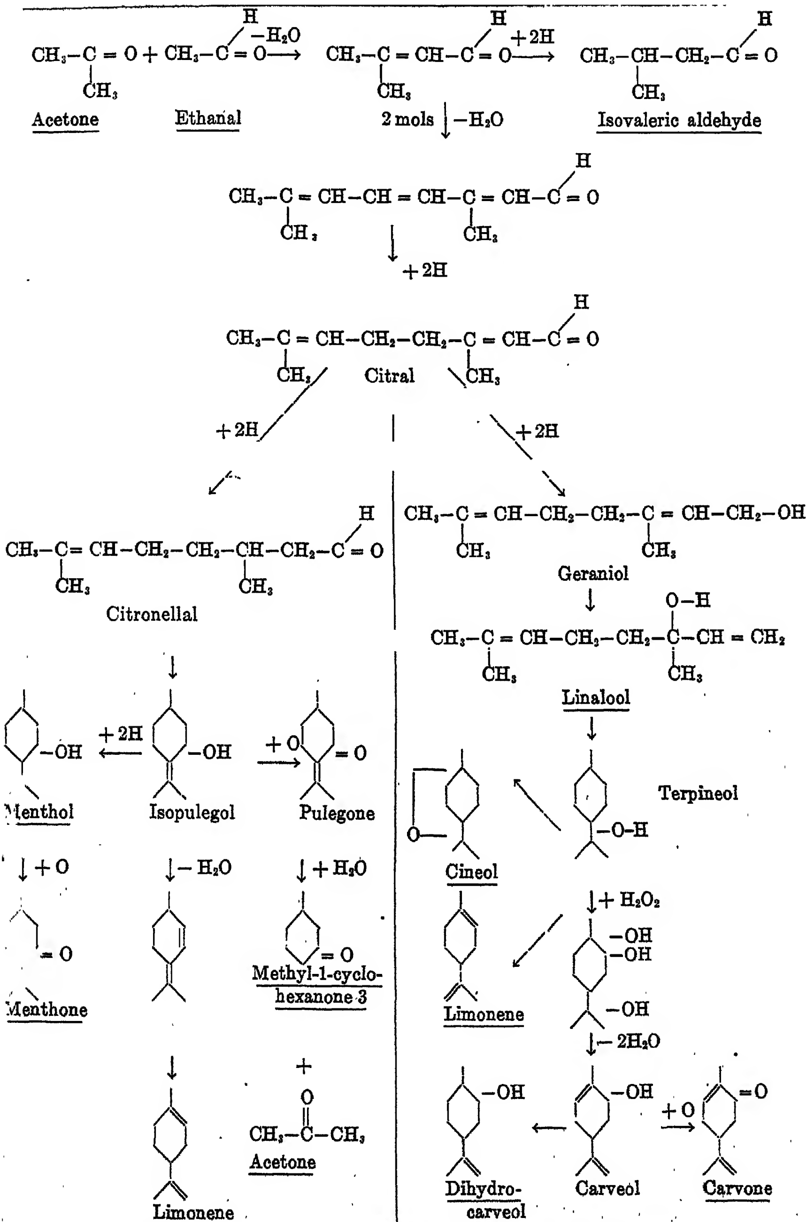
"American mint."

Mentha piperita, var. officinalis, forma rubescens, Camus.

A European oil reported to have a pulegone odor; one from Florida, that of linalool.	Acetaldehyde.	Acetic acid.
	Acetone.	Butyric acid.
	Methyl alcohol.	1-Limonene.
	Furfural.	Phellandrene.
	Isovaleric aldehyde.	Carvone.
	Amyl alcohol.	Dihydrocarveol (ester).
	Acetic acid.	Cineol.
	Isovaleric acid.	
	Pinene.	
	Phellandrene.	
	Cineol.	
	1-Limonene.	
	Menthol.	
	Menthone.	
	Methyl-1-cyclohexanone-3.	
	Menthyl esters.	
	Cadinene.	
	A lactone.	
	Dimethyl sulfide.	

[6] *Univ. Wisconsin Pharm. Exp. Station, Circular 2*, October, 1920, 17.

[7] Gildemeister, E., Die Aetherischen Oele, Leipsic, 2nd edition, 1910, iii.



Peppermint (Mentha piperita) *Spearmint (Mentha spicata)*

Compounds known to occur in the oils are underlined

This scheme or picture of how the oils of spearmint and peppermint may be elaborated offers, in spite of its tentative nature, numerous suggestions for speculation and experiment. Thus the striking parallelism in the relations of analogous compounds, for example of menthol and pulegone to isopulegol in the one mint and of dihydrocarveol and carvone to carveol in the other unmistakably suggests a common mechanism of formation. But most interesting is the thought that the Mendelian "factor," if such it is, which governs the formation of the carvone group in spearmint and of the menthone group in peppermint, lies in the conditions affecting the reduction of citral. For, as outlined, the two groups are separated at that point by a common type of reaction. And it is easy to conceive that the conditions which caused the reduction of the aldehyde group in spearmint were modified in cross-breeding to bring about the reduction of a carbon to carbon double bond in peppermint.

CONCLUSIONS.

Although it was found that the oils of American and Japanese peppermints are not strictly comparable in a botanical sense, still some interesting results are evident as a result of studying the problems originally suggested by that idea.

The constituents of the volatile oils of *Mentha piperita, var. officinalis* and *Mentha spicata* Hudson have been listed and compared.

A possible scheme of the biogenesis of the most important constituents has been drawn up.

It is evident that a great similarity exists in the reactions by which the menthol group on the one hand and the carvone group on the other are elaborated.

The two groups may have a common precursor, and are each derived from it by a reaction involving the addition of two atoms of hydrogen—the difference being only in the point of reduction.

The urgent need of a reliable examination of the volatile oil of *Mentha aquatica* becomes evident.

It should finally be emphasized that these data are presented only as a preliminary survey of what is known and as a glimpse of what may be. Also it is desired to emphasize the opportunity existing in the study of these common plants for the elucidation of phenomena which may be hereditary in character. The work is being continued.

THE CHEMISTRY OF THE OXIDATION OF SULFUR BY MICROORGANISMS TO SULFURIC ACID AND TRANSFORMATION OF INSOLUBLE PHOSPHATES INTO SOLUBLE FORMS.*

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(Received for publication, October 13, 1921.)

Oxidation of Sulfur by Microorganisms.

When sulfur is added to unsterile soil, it is slowly oxidized to sulfuric acid; when the soil is previously sterilized, oxidation of the sulfur takes place only to a very limited extent depending upon the other chemical substances present. But when a sulfur-oxidizing organism is introduced, the sulfur is rapidly oxidized to sulfuric acid. This acid acts upon insoluble soil constituents such as calcium and magnesium carbonates, calcium silicates, and tricalcium phosphate, and brings them into solution. This process has been utilized by Lipman and associates (1916) for the transformation of the insoluble tricalcium phosphate into soluble forms by composting rock phosphate, sulfur, and soil to which the sulfur-oxidizing bacteria have been added. A few principles involved in these transformations, both by crude and pure cultures of the sulfur-oxidizing organisms, are set forth in this paper.

As a result of a series of studies, several organisms have been isolated, which are able to oxidize sulfur under various conditions. The oxidation of sulfur under acid and alkaline conditions seems to be affected by different groups of microorganisms. A detailed study of occurrence, morphology, and physiology of

* Technical Paper No. 54 of the New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

these organisms is found elsewhere (Waksman and Joffe, 1920–22). One of the most interesting organisms isolated by the authors is an aerobic, autotrophic, minute bacterium, *Thiobacillus thiooxidans* Waksman and Joffe, which is able to oxidize sulfur to such an extent as to reduce the hydrogen ion concentration of the medium to a pH of less than 1.0, even in the presence of buffering materials. It derives its energy from the oxidation of the sulfur and the carbon from the CO_2 of the atmosphere. The nitrogen can be supplied in the form of inorganic or organic materials.

In taking up the chemistry of the sulfur oxidation, attention must be called to the aerobic nature of the phenomenon.

$$\underset{64}{2S} + 2H_2O + \underset{96}{3O_2} = \underset{196}{2H_2SO_4}$$

Thus, for 64 units of sulfur, 96 units of oxygen are required to produce 196 units of sulfuric acid. The effect of oxygen is, therefore, of prime importance in the oxidation of sulfur.

Experiments with crude cultures of the organism reported elsewhere (Joffe, 1922) substantiate the theoretical suppositions based on the empirical equations involved in the chemistry of sulfuric acid. Mixtures of sulfur, rock phosphate, and soil inoculated with crude cultures of sulfur-oxidizing organisms were prepared; one set was aerated and the other left unaerated. The amount of phosphates brought into solution and the change in the hydrogen ion concentration, as expressed by the exponent pH of Sörensen, were used as criteria. The aerated mixtures were leading and, after 100 days, the percentage increase of soluble phosphates in the aerated over the non-aerated was 6 per cent, with a similar correlation in the increase of the hydrogen ion concentration. It is interesting to record here the fact that this biological process follows the laws of inorganic reactions. According to the mass law, the velocity of any reaction depends on the mass of the active ingredients involved and is at any moment proportional to the molecular concentration of the reacting components and a constant, which is characteristic of the chemical nature of the reacting substances. Whatever transformations the oxygen undergoes in the metabolism of the organism, the end-product is sulfuric acid; an increase in oxygen tension increases

the mechanism of oxidation of sulfur by the organisms. It is also possible that the oxygen from the air is not the only source; as pointed out above this particular organism derives its energy not from carbohydrates but from the oxidation of sulfur and is autotrophic in nature. Like green plants, the autotrophic organisms use the carbon dioxide from the air for structural purposes, but, unlike plants, these organisms accomplish it without the intervention of the photochemical reactions. The process of assimilation of carbon dioxide is accompanied by the splitting off of oxygen, which may also be used by the sulfur organisms in the process of oxidation.

Oxidation of Sulfur in the Ordinary Cultivated Soil.

Several typical experiments will be reported here to illustrate the mechanism of sulfur oxidation in the soil, both in the absence and in the presence of small and large amounts of rock phosphate. The sulfur and phosphate were added to the soil and well mixed. A crude well developed culture was used for inoculation. The moisture content of the soil was kept at an optimum by the addition of water at weekly intervals. The cultures were incubated at 25–27°C. The pH values were determined colorimetrically, according to the method of Clark and Lubs (1917); the phosphates and sulfates according to the method of the Official Agricultural Chemists (1916).

The results tabulated in Table I represent the oxidation of small amounts of sulfur in the soil. In this case 22.5 mg. of sulfur and 90 mg. of rock phosphate were added to 600 gm. of soil. The results tabulated in Table II represent the oxidation of large amounts of sulfur when introduced with large amounts of rock phosphate into the soil. In this case 30 gm. of sulfur and 90 gm. of rock phosphate were mixed with 480 gm. of soil in small pots.

When the course of change in reaction due to the sulfur oxidation, in the presence of tricalcium phosphate is studied, we find that the curve is regular till the pH reaches 2.8, then it becomes flat. This is a crucial point and, as long as there will be any phosphate left undissolved, the reaction will not go down very much, since at that point all the acid formed from the oxidation of the sulfur is used not in increasing the reaction of the medium, but in transforming the rock phosphate into soluble form. Once

the phosphates have been made soluble, the acidity begins to increase. This will be made clearer in the discussion of the sulfur oxidation by pure cultures in solution.

TABLE I.

The Oxidation of Small Amounts of Sulfur in the Soil.

Period of incubation.	pH value.
days	
0	6.2
3	6.2
9	6.2
15	6.0
22	5.8
29	5.6
39	5.6
56	5.2
70	5.2
102	5.2

TABLE II.

The Oxidation of Large Amounts of Sulfur in the Soil in the Presence of Large Amounts of Rock Phosphate.

Period of incubation.	pH value.	Soluble sulfates in 1 gm. of soil.	Citrate-soluble phosphates in 1 gm. of soil.
days		*mg. of SO_4*	*mg. of P*
0	6.2	0.95	2.83
3	6.2	0.96	2.83
9	5.0		
15	3.4	3.60	4.28
22	3.2	20.80	7.13
29	3.0		
39	3.0	30.80	14.76
56	2.2	35.25	20.67
85	2.0		
102	1.8		19.58

Oxidation of Sulfur in Solution by Thiobacillus thiooxidans.

When a proper medium is used, with sulfur as the only source of energy, the pure culture of the organism rapidly oxidizes the sulfur to sulfuric acid. To prevent a rapid change in reaction

buffering substances are used. The course of reaction depends chiefly upon the nature of the buffering agents. When soluble phosphates are used, the curve is more or less continuous; when insoluble phosphates are used the curve has a definite flat portion at a pH of 2.6 to 2.8, the point at which the insoluble phosphates become soluble, and, only after all the phosphate has gone into solution, the curve rises again. When more insoluble phosphate is added at this point, the curve reaction will be kept at the pH of 2.8 to 2.6, till all the insoluble phosphate has disappeared. The medium used for this experiment consisted of sulfur, 10 gm.; $(NH_4)_2\ SO_4$, 2 gm.; $MgSO_4$, 0.5 gm.; $FeSO_4$, 0.01 gm.; KH_2PO_4,

TABLE III.

The Oxidation of Sulfur by Pure Culture of Thiobacillus thiooxidans.

Period of incubation.	No $Ca_3(PO_4)_2$.	0.5 per cent $Ca_3(PO_4)_2$.	Gradual addition of $Ca_3(PO_4)_2$.*
days	*pH*	*pH*	*pH*
0	4.4	5.0	4.4
3	4.4	5.0	4.2
7	3.2	4.4	3.2*
11	2.2	2.8	2.8
17	1.6	2.6	2.6*
23	1.6	2.4	3.2
33	1.4	2.1	3.0
52	1.2	1.8	2.8

* 0.5 gm. of $Ca_3(PO_4)_2$ has been added per 100 cc. of medium.

5 gm.; distilled water to make 1,000 cc. When $Ca_3(PO_4)_2$ is not used, 0.25 gm. of $CaCl_2$ has been added per 1,000 cc. of medium. The medium was placed, in 100 cc. portions, in 250 cc. Erlenmeyer flasks and sterilized on 3 consecutive days in flowing steam. The flasks were then inoculated with a pure culture of *Thiobacillus thiooxidans* by means of a loop, and incubated at 25–27°C. The results are presented in Table III.

It has been pointed out elsewhere (Waksman and Joffe, 1921) that the optimum reaction for the activities of *Thiobacillus thiooxidans* lies at a pH of 3.0 to 4.0. If the reaction of the medium is less acid, the reaction changes in the beginning only very slowly, but, once the optimum is attained, the curve rises rapidly.

A detailed experiment is next reported which will show definitely the relation between the sulfur oxidation, as demonstrated by change in reaction, accumulation of sulfates, and the transformation of phosphates, as shown by the amounts of soluble phosphates and calcium.

A medium containing 2 gm. of $(NH_4)_2SO_4$, 0.5 gm. of $MgSO_4$, 5 gm. of KH_2PO_4, and a trace of $FeSO_4$ per liter, was placed, in 400 cc. portions into fifteen 1 liter Erlenmeyer flasks. 3 gm. of $Ca_3(PO_4)_2$ and 4 gm. of powdered sulfur were added to each flask. The flasks were inoculated with 1 cc. portions of a pure culture of

TABLE IV.

Course of Sulfur Oxidation as Indicated by Change in Reaction and Amount of Soluble Sulfates, Phosphates, and Calcium in the Culture Solution.

Age of culture.	pH	Phosphates in 100 cc. of solution.	Sulfates in 100 cc. of solution.	Calcium in 100 cc. of solution.
		mg. of P	*mg. of SO_4*	*mg. of Ca*
Control.	6.0	123	230	17.4
20 hours.	6.0		230.4	17.44
70 "	5.4			
88 "	4.9	125.26	248.0	24.74
110 "	3.5	123.20	260.15	26.85
134 "	3.0	200.06	322.2	31.0
6.5 days.	2.6	171.64	366.4	64.2
8.5 "	2.6	210.04	498.8	118.8
10.5 "	2.5	255.46	511.4	104.9
13.5 "	2.3	350.00	450.6	101.4
19.5 "	2.1			81.6
34 "	1.3			

Thiobacillus thiooxidans and incubated at 25–27°C. At various intervals, small amounts of the liquid were taken out from four to six flasks, and determinations made of the pH value, of the sulfates, phosphates, and calcium in solution. The results based on the average of four to six determinations, are tabulated in Table IV.

The course of sulfur oxidation is best followed by the change in the hydrogen ion concentration of the medium (pH value). Of course, where there are large amounts of buffering agents or insoluble carbonates or insoluble calcium phosphate, a much larger

amount of sulfur will have to be oxidized to bring about a definite change in the pH value. The amount of sulfur oxidized has been reported in Table IV as sulfates; it may be pointed out here that in practically all cases the sulfur oxidized, as indicated by the amount of residual sulfur in solution, has been almost quantitatively transformed into sulfates. The phosphates and calcium columns in Table IV will be discussed below.

Transformation of Insoluble Phosphate into Soluble Forms.

The reactions involved in the conversion of rock phosphate (insoluble tricalcium phosphate) into soluble forms (di- and monocalcium phosphate and phosphoric acid) by means of acids belong to the type of reactions of heterogeneous systems. The rock phosphate minerals have no definite composition and the products formed are not always definite. In such heterogeneous systems the speed of the reaction is a function of a greater number of variables than in the case of a homogeneous system. According to Kazakov (1913), there are some factors which are common to both systems and these are: (1) concentration of the reacting mass; (2) temperature of the reacting medium; (3) the amount of contact of the reacting substances; (4) the speed of diffusion of the reacting substances; and (5) catalytic agents.

Besides these factors we have others in a heterogeneous system where solid solution phases occur. These are: (1) the size of contact surface;[1] (2) chemical composition of the solid phase; (3) the physical properties of the solid phase; and (4) the influence of formation of a solid phase as a result of the reactions.

The factors; chemical composition of the solid phase, and the physical properties of the solid phase; have a tremendous influence on the speed of the reaction and they are the least known, since the chemical make-up of the rock phosphate is still obscure.

[1] The size of the particles of the rock in the manufacture of acid phosphate has an important influence. Theoretically, all other conditions being equal, the speed of solution of a solid in a liquid is proportional to the contact surface and in circular bodies (as we would suppose in finely powdered rock phosphate) the surface is proportional to the square of the radius; then, particles with a radius of 0.1 mm. will dissolve twenty-five times faster than particles with a radius of 0.5 mm.

The process on the transformation of insoluble phosphates has been the subject of study by a number of investigators in this country and in Europe. We may merely refer to the work of Cameron and Bell (1907) of the Bureau of Soils, of Schucht (1909), Meyer (1905), Stoklasa (1911), Kazakov (1913), and others.

According to Kazakov (1913), the scheme of reactions involved in the formation of soluble phosphates are:

No.	H_2SO_4	Resultants obtained.	
		Liquid phases.	Solid phases.
1	When added in excess.	H_3PO_4 + H_2SO_4 + sulfates of Ca, Al, and Fe.	$CaSO_4$
2	Close to optimum.	H_3PO_4 + sulfates of Ca, Al, and Fe.	$CaSO_4$
3	Optimum.	H_3PO_4 + sulfate of Ca + phosphates of Al and Fe.	$CaSO_4$
4	Not enough acid.	H_3PO_4 + sulfate of Ca + phosphates of Ca, Al, and Fe.	$CaSO_4$ + part of undissolved phosphate.

Before we go into a discussion of the scheme, we shall take up the experimental results of the transformation of the tricalcium phosphate into soluble phosphate through the oxidation of sulfur by *Thiobacillus thiooxidans.*

The culture medium given above has been used, with a slight modification: the KH_2PO_4 was reduced to 1 gm. per liter and 1 gm. of $Ca_3(PO_4)_2$ was added to each flask containing 100 cc. of medium. The medium was sterilized in flowing steam on 3 consecutive days, 30 minutes each day, then the flasks were inoculated with *Thiobacillus thiooxidans* and incubated at 27°C. Only the pH values and water-soluble phosphates (in solution) are reported in Table V and Fig. 1. The results are based on averages of four to six flasks.

The column of soluble sulfates is of extreme interest. At a pH of 2.6, a sudden rise in the amount of soluble phosphates takes place after the soluble sulfates have reached a maximum. This is in accordance with the scheme suggested by Kazakov. Up tc the point of pH = 2.6 to 2.7, the liquid phase consists of monocalcium phosphate and gypsum, we therefore have a large amount

of soluble sulfates. However, as soon as more sulfuric acid is formed through the oxidation of sulfur, the monocalcium phosphate is attacked first, since in any reaction the liquid phase comes in first; the products formed are phosphoric acid and gypsum.

$$CaH_4(PO_4)_2 + H_2SO_4 + 2H_2O = CaSO_4 \cdot 2H_2O + 2H_3PO_4$$

The $CaSO_4 \cdot 2H_2O$ is soluble to a certain extent in phosphoric acid, but is forced out from solution because of the fact that the phosphoric acid reacts with the remaining tricalcium phosphate, forming again monocalcium phosphate until the reaction comes to an equilibrium forming gypsum and phosphoric acid. With the

TABLE V.

Course of Sulfur Oxidation and Transformation of Insoluble Phosphates.

Age of culture.	pH	Soluble sulfates in 100 cc.	Soluble phosphates in 100 cc.
		mg. of S	*mg. of P*
Control.	5.4	68.39	45.57
1	5.4	67.64	42.61
2	5.3	69.70	47.20
4	4.6	73.79	55.00
6	3.5		
8	2.6	152.53	103.56
10	2.6	109.7	93.00
12	2.6	78.54	186.30
15	2.4	87.6	207.28

accumulation of the phosphoric acid, more gypsum goes in solution and the soluble sulfates increase again. The continuous increase of the insoluble sulfates after all of the tricalcium phosphate goes in solution is then due to the further oxidation of sulfuric acid.

The column of soluble phosphates also proves the mechanism of the process suggested by Kazakov. Here also we find a gradual increase of the soluble phosphates, since the amounts of sulfuric acid in the early part of the incubation period is small. As soon, however, as the pH reaches 2.6 to 2.7, which is the crucial point of the reaction, the soluble phosphates increase rapidly. Practically all of the tricalcium phosphate goes into solution in 2 days after the crucial point is reached.

The course of conversion of insoluble phosphates in composts of rock phosphate and sulfur has been taken up by Joffe (1922).

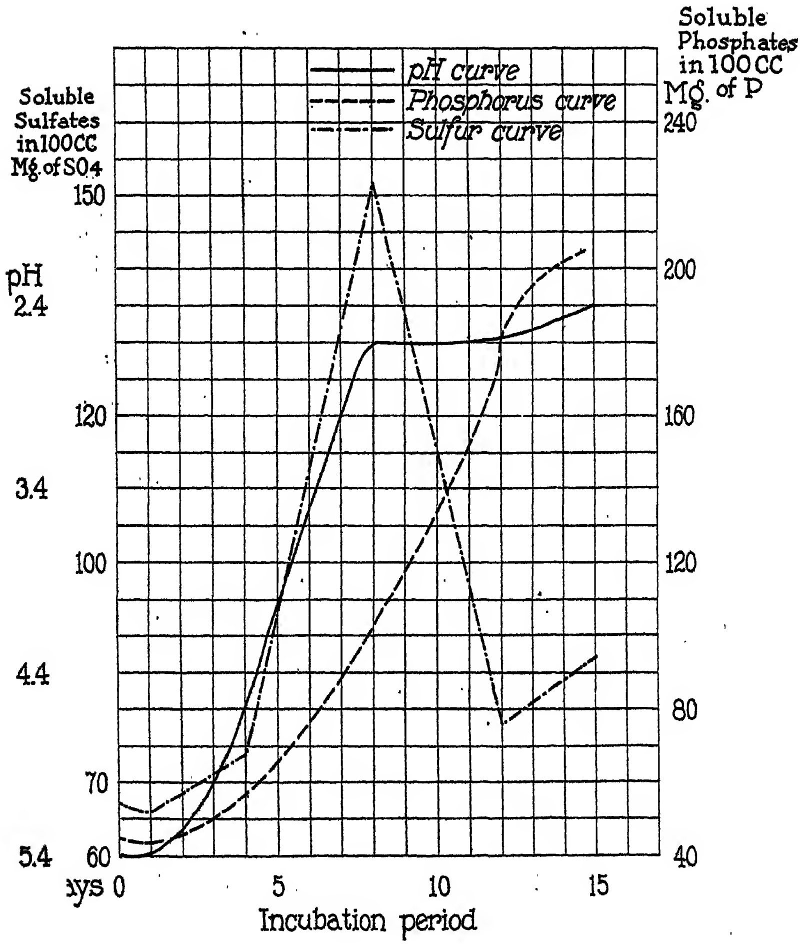

FIG. 1. Change in reaction and increase of soluble sulfates and phosphates by pure culture of *Thiobacillus thiooxidans*.

In this case the reactions are not so apparent, since the indefinite chemical make-up of the raw phosphates introduces a great number of side reactions.

SUMMARY.

1. The curve of sulfur oxidation both in the soil and in solution by pure and impure cultures of *Thiobacillus thiooxidans* is a growth curve.

2. The mechanism of sulfur oxidation to sulfuric acid by *Thiobacillus thiooxidans* obeys the laws of inorganic catalysis.

3. The transformation of insoluble rock phosphate to soluble phosphates by the sulfuric acid formed from the oxidation of sulfur by *Thiobacillus thiooxidans* is similar to the process taking place in inorganic reactions.

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CHANGES IN THE REFRACTIVE INDEX OF THE BLOOD SERUM OF THE ALBINO RAT WITH TEMPERATURE.

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It is a well known fact that changes in the temperature of a solution induce changes in its refractive index (1). In as far as dilute solutions of proteins are concerned it is apparent from the studies of Robertson (2) that these changes are due to changes of the refractive index of the solvent, usually water. This effect on the solvent must be taken into account when measurements of the refractive index are made at different temperatures. Non-recognition of this fact invalidates not a few papers dealing with studies of the refractive index of blood serum. Observations of the temperature variation of this laboratory for over a year have shown an extreme range of 15°C. In view of this fact it became necessary to determine the temperature correction factor for blood serum of the albino rat for use in connection with some refractometric studies now under way.

The sera for examination were obtained from mature albino rats. The animals were etherized and the heart was exposed. A cut was made in the ventricular wall and blood from the beating heart was collected in small test-tubes. These were immediately tightly corked and allowed to stand for 2 or 3 minutes when coagulation was complete. The clot was broken up with a fine glass rod and the serum was separated from the fibrin and corpuscles by centrifuging for ½ hour. At the end of this period the supernatant serum was poured into another small test-tube and again centrifuged for a like period. During the process of centrifuging the tubes were tightly corked to prevent loss of water by evaporation. There resulted from this procedure a clear serum which was transferred to the cell of the refractometer by means of a pipette. After mixing, a portion was taken for the

determination of the water content. The cap of the cell was then lowered into place and the cell was sealed with paraffin having a melting point of 58–60°. This prevented undue loss of water from the sample under examination. The instrument used for these tests was a Pulfrich refractometer made by Carl Zeiss at Jena. Readings were made to tenths of a minute. The instrument was connected with the temperature regulating apparatus described by Reiss (3). Since much of the work reported here was carried on in the winter months the water coming in from the outside contained considerable dissolved air which was liberated on warming and accumulated in the bend of rubber tubing connecting the warming chamber of the cell with the outflow. This interrupted the even flow of water through the instrument and resulted in distressing irregularities of temperature. These were eliminated by inserting into the system a small bottle with a three-hole rubber stopper. Into the central hole there was placed a long glass open tube extending above the level of the tank from which the water is supplied. The other two holes served to connect the bottle with the system. The bottle was placed in the part of the system between the warming coil and the refractometer. With this addition to the apparatus most of the bubbles of air were caught before getting into the refractometer and escaped through the glass tube. When the cell had been sealed a reading was taken of the angle of refraction and of the temperature—usually around 17°—of the enclosed serum. The temperature of the cell and contents was then increased by warming the water circulating through the apparatus. The attempt was made to raise the temperature by steps of 1°. When the new temperature level had been reached and maintained for at least 1 minute another reading was made. This procedure was repeated until the desired maximum temperature had been reached, usually around 35°. The apparatus was then rapidly cooled to approximately the temperature at the beginning of the examination and a final reading was taken. This gave the effect of the heating on the refractive index of the serum constituents. The cell was then opened, a sample of serum removed, and its water content determined. Thus any loss of water from the serum by vaporization during the examination was determined.

As Hatai (4) has shown, the value of the refractive index of the serum of the albino rat varies with the age and to a less degree with the size of the animal. The animals in this series varied somewhat in size although they were all of about the same age. These differences in size resulted in differences in the initial refractive index of the serum. In order that the values might be brought to a common basis for purposes of study the percentage difference between the refractive index of the serum and that of water at the initial temperature of observation was determined. The subsequent observed indices for the serum at the different temperatures were multiplied by this factor, thus making the curve of the change in refractive index with temperature of the serum comparable with the temperature curve of water, the solvent. Any changes in the refractive index due to the influence of temperature on the serum constituents other than water would then be shown by a deviation of the curve for the serum from that for water. The results of the observations reported in this paper were obtained from seventeen sera.

When the calculation noted above had been made it was seen that the sera fell into two groups with respect to their accommodation to the water curve. In the first group there were eight sera. These had been obtained from rats early in the winter. In the second group there were nine series of observations. These had been made during the early spring. The averages of the calculated refractive indices for each degree of temperature were determined for each group and are plotted in Charts 1 and 2, using the curve of the refractive index of water on temperature as the norm.

Chart 1 shows conclusively that in this group (1) of sera the changes in the refractive index with temperature are solely due to the changes in the refractive index of the solvent water. This holds up to a temperature of about 29°. Above this point there is an indication of a tendency for the refractive index of the serum to increase more than does that of water. This curve tends to support the contention of Robertson (2) that when the solvent change is considered the influence of proteins in solution on the refractive index is independent of the temperature between 20 and 40°. In Group 2, however, the curve of which is plotted in

Chart 2, it is seen that the refractive index tends to fall regularly away from that of water with the rise in temperature. The reaction of this group fails to support Robertson's belief mentioned

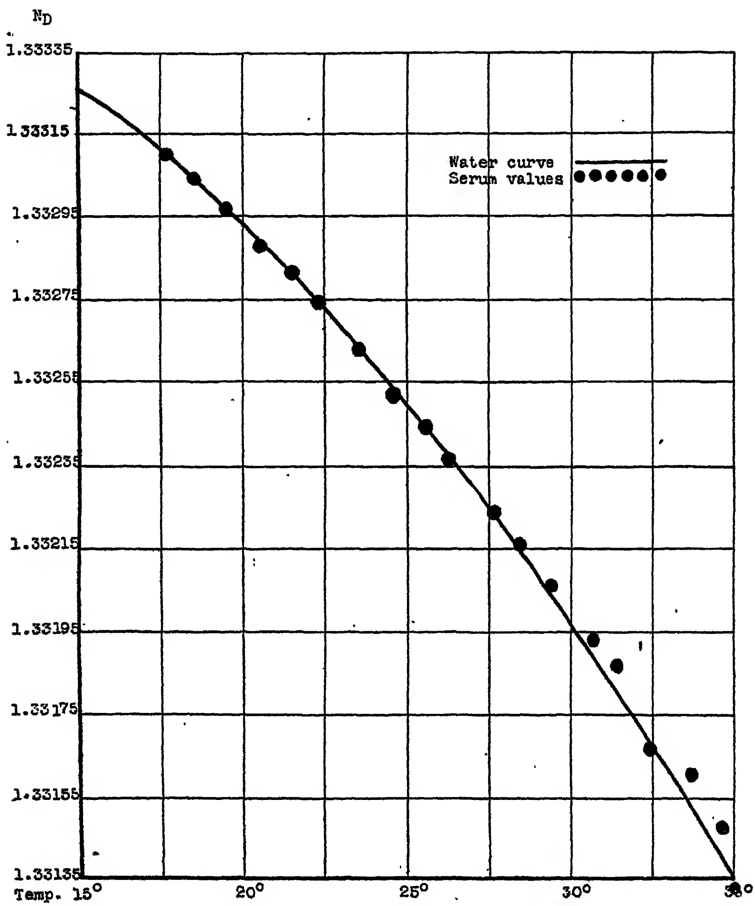

CHART 1. Showing the refractive index of blood serum of the albino rat with increasing temperature. Group 1, winter rats .... Serum values. ——— Water curve.

above. This discrepancy may possibly be explained by the fact that the concentration of protein material in blood serum is considerably above the concentrations used by Robertson in his

temperature studies. This greater concentration may bring into relief differences which in smaller concentrations would be within

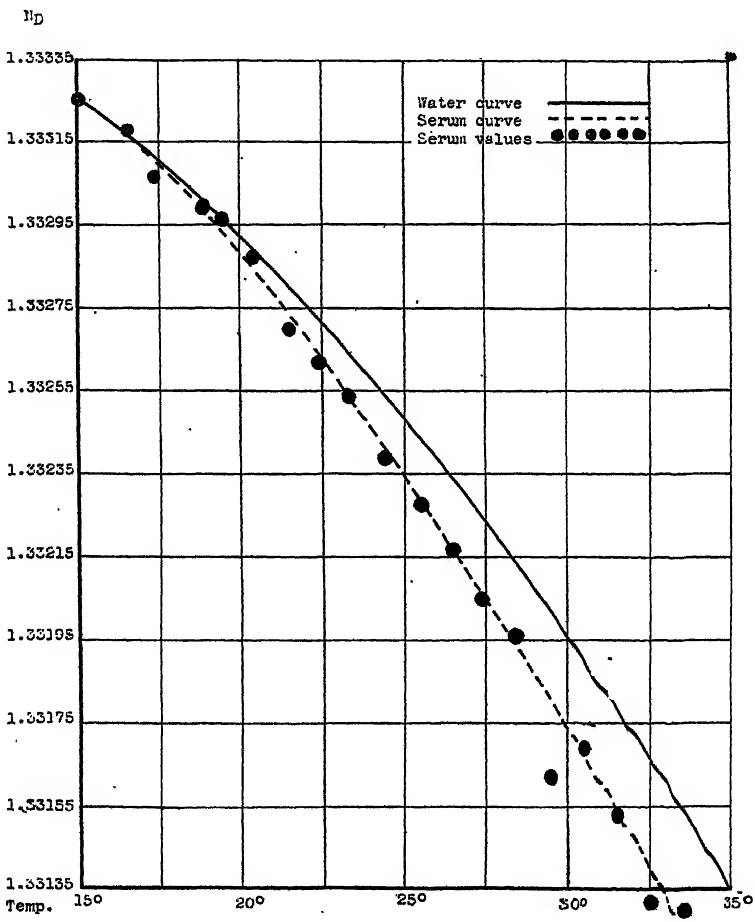

CHART 2. Showing the refractive index of the blood serum of the albino rat with increasing temperature. Group 2, spring rats .... Serum values. ———— Serum curve. ———— Water curve.

the limit of error of the method of observation and hence unnoticeable. The difference in the temperature effect on the two groups is statistically valid as will be shown presently.

In Table I there is given the mean, standard deviation, and probable error of the mean of the body length, body weight, and water content of the sera in both groups before and after the refractometric examination. The table shows that none of these are factors in the difference in behavior of the sera towards changes in temperature. Nor can the difference be attributed to a general difference in the state of digestion and absorption, since at least 18 hours regularly intervened between the last feeding and the time of the taking of the blood. The only factor at present indicated is a possible seasonal variation, since the determinations of the first group were made on winter rats and those of the second group on spring rats. Although we would not care to stress this point, when the fact that in the spring there is an increased sexual activity in the rat is correlated with the

TABLE I.

	Group 1.			Group 2.		
	Mean.	Standard deviation.	Probable error of mean.	Mean.	Standard deviation.	Probable error of mean.
Body length, *mm*.	188	12.5	3.4	184	15.1	3.6
Body weight, *gm*.	183	26.6	7.3	180	50.1	11.9
Water, before, *per cent*.	92.2	0.51	0.13	92.2	0.25	0.06
Water, after, *per cent*.	92.0	0.45	0.11	92.0	0.45	0.10

observation of Hatai (4) that irregularities in the course of the curve of the refractive index of the serum of the albino rat on age occur around the period of puberty, it would appear as if an interpretation based on such a seasonal modification is strengthened.

Whatever the determining factor may be, it is evident that in some sera certain constituents other than the solvent water are so influenced by temperature changes that they give rise to changes in the refractive index entirely apart from those produced by the solvent.

Robertson (5) has stated that the change in the refractive index of a solvent is a function of the size of the molecules of the solute. This, of course, refers to conditions at uniform temperature. It is not improbable that the state of the colloidal equilib-

rium existing in those sera showing regular deviations from the water curve with rising temperature may be relatively unstable and that as a result of the changes in temperature, changes in the degree of dispersion of the colloidal constituents take place. Such an assumption is supported by Robertson's (6) observation that as protein solutions approach the point of coagulation there occurs a decrease in the refractive index. It should be noted in this connection that this change is not rapidly reversible since when the serum is cooled to the point where the initial reading was taken, the value of the refractive index is usually somewhat greater than it was at the beginning of the examination.

Turning now to the practical application of these results, there is given in Table II, the mean, standard deviation, and probable error of the mean of the change in reading of the angle of refraction for 1° of temperature for the two groups. It is seen that in the first group each rise of 1° is accompanied by a corresponding increase of approximately 1 minute in the angle of refraction. In the second group the increase of the angle of refraction for each degree of temperature is 1.4 minutes, a somewhat greater value. This difference between the two groups is statistically valid and substantiates the curve in Chart 2.

TABLE II.

Statistical Values of the Angles in Minutes of Refraction of the Two Groups.

	Group 1.	Group 2.
	minutes	*minutes*
Mean	1.08	1.41
Standard deviation	0.23	0.36
Probable error of mean	0.02	0.03

Notwithstanding the difference, an inspection of the index of variability shows that for all practical purposes the mean of the change in the angle of refraction of the two groups—1.25 minutes—for each degree of temperature is an acceptable factor and gives results accurate within plus or minus half a minute of refraction. This can be used for correction of the observed readings. Taking 20° as the standard temperature, when a refractometric reading is made of blood serum at a temperature above

this value, 1.25 times the difference between 20° and the observed temperature should be subtracted from the observed reading. If the temperature is between 17.5 and 20° the difference times the factor is added. The following formulas are simple expressions of this relation; $I = i - 1.25(t - 20°)$ and $I = i + 1.25(20° - t)$ where I is the corrected angle of refraction; i the observed angle of refraction; and t the observed temperature.

SUMMARY AND CONCLUSIONS.

A study of the changes in the refractive index of the blood serum of the albino rat with rising temperature showed that two types can be distinguished according to the nature of the response. In the first type the changes in the refractive index coincide with those of the solvent water and can be attributed to this serum constituent. In the second type the curve of the change of refraction with rising temperature falls away from that of water. This demonstrates a participation in the response of serum constituents other than the solvent water. The causes of this difference are unknown, although there is a possibility that a seasonal variation may be a determinant. It is certain that in this series the factors of body length, body weight, age, and water content of the serum both before and after the experiment, and previous state of digestion and absorption are not the causes of the difference between the two groups.

The correction for the reduction of the observed angle of refraction to the common base at 20° when readings are taken at different temperatures is obtained by use of formulas given in the text. These formulas hold for temperatures between 17.5 and 35°C.

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COLORIMETRIC DETERMINATION OF URIC ACID.

ESTIMATION OF 0.03 TO 0.5 MG. QUANTITIES BY A NEW METHOD.

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Determinations of uric acid, which depend upon the direct weighing of the substance as such or upon an estimation of the nitrogen content of an insoluble salt, have been largely replaced by methods which quantitatively measure its oxidation. Any of these procedures, whether volumetric or colorimetric, are useful in proportion to their success in (*a*) separating the uric acid from other substances which might give a value in the oxidation reaction and in (*b*) making the actual conditions of the reaction as specific as possible for uric acid.

Volumetric methods, in most cases, have not been sufficiently sensitive for satisfactory application to less uric acid than is found in 100 cc. of urine. Also the means for preliminary precipitation of uric acid made use of in these methods were crude. One of us[1] determined conditions for a complete precipitation of uric acid in any amount with zinc salts, and found conditions under which permanganate oxidation could be used successfully on such quantities of uric acid as are found in 5 cc. of urine.[2] The much smaller amount of uric acid found in blood is below the limit of accuracy which it is possible to attain with the zinc precipitation-permanganate oxidation procedure.

Folin and Macallum[3] recognized the need of separation of uric acid from interfering substances and attempted the removal of polyphenols from urine residues before oxidizing uric acid with

[1] Morris, J. L., *J. Biol. Chem.*, 1916, xxv, 205.

[2] Morris, J. L., *J. Biol. Chem.*, 1919, xxxvii, 231.

[3] Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912–13, xiii, 363

alkali phosphotungstate. Folin and Denis[4] improved this separation by their adaptation of the Salkowski[5] precipitation of uric acid to precede the phosphotungstate oxidation. The method described in this paper makes use of the zinc salt separation of uric acid which has proved very satisfactory in our hands and determines the uric acid so separated by a new colorimetric procedure which possesses the double advantage of greater specificity and greater color obtainable for unit weight of uric acid. With slight variations it is equally applicable to urine and blood. In the latter case we have removed the proteins by the tungstic acid precipitation of Folin and Wu[6] and found the procedure satisfactory for our purpose.

The results which we have obtained with the new method are, we believe, more dependable and uniform than the usual ones obtained with any of the procedures based upon silver precipitation. One cause for irregularities in the results obtained by means of the silver methods is inherent in the metal used. Silver solutions, even when protected from light, soon become clouded with, and eventually precipitate, a form of "reduced silver." The acid silver lactate solution used by Folin and Wu,[6] is noticeably cloudy soon after preparation and develops a heavy precipitate on standing. The ammoniacal silver magnesium solution of Benedict and Hitchcock[7] also forms a scum around the neck of bottles, spouts of dropping bottles, etc., which occasionally contaminates the reagent added and thus gives erroneous results. This "reduced silver," when taken up in sodium cyanide and the mixture made alkaline with sodium carbonate and phosphotungstic acid reagent added, gives a deep blue color even when taken in small amounts. (The color can also be developed by reversing the order of these reagents.) This possibility of the presence of such a substance is sufficient to cast doubt upon any determination which makes use of a silver precipitation. It is evident that the urine procedure of Folin and Wu[8] is particularly

[4] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 469.

[5] Salkowski, E., *Virchows Arch. path. Anat.*, 1870, lii, 58. Ludwig, E., *Wien. med. Jahrb.*, 1884.

[6] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

[7] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 459.

open to criticism on this point for the reason that the entire precipitate is dissolved in sodium cyanide previous to the development of color. The extraction step of their blood procedure to a large extent eliminates this error. Myers'[9] suggestion in regard to this method that cyanide be added before the second centrifuging can hardly be accepted as an improvement when considered in the light of these facts. We have found that the reliability of these methods is greatly increased when care is taken to use only perfectly clear silver reagents.

Another possible source of error in the Folin and Wu procedure is a common impurity found in many of the best grades of sodium sulfite we could obtain. Three out of four lots of this salt, from as many sources, gave a reaction with the uric acid reagents. The color which developed in each instance was sufficient to introduce a considerable error if the sulfite had been used in the amount prescribed for the determination.

Neither silver nor sulfite is used in the new method described in this paper. The new method requires the use of no metal which may exist in a reduced form or reagent which is likely to contain any interfering impurity. Fortunately, in addition to their adaptability to the purpose, there is the additional advantage that all the reagents used are relatively inexpensive.

The use of potassium cyanide suggested by Benedict and Hitchcock[7] for tying up the silver greatly improved the earlier Folin-Denis[4] method. We successfully applied cyanide for the purpose of forming a double radical with zinc and found it useful as well in two other particulars. Used in larger amounts its alkalinity is sufficient for the complete and rapid development of the color when the actual molecular concentration of alkali is still less than one-third that used in the Benedict-Hitchcock procedure. The second advantage in the use of cyanide is the very marked increase of color which is obtained from a given amount of uric acid.

That this increase is due to the uric acid reduction of phosphotungstate, while the cyanide only accelerates the reaction, is shown by results of the kind typified in Table I. The negative result of Flask 10 shows that the cyanide will not act directly on the phosphotungstate. The 18 per cent increase of color in

[9] Myers, V. C., *J. Lab. and Clin. Med.*, 1919-20, v, 499.

TABLE I.

Effect of Sodium Cyanide upon Phosphotungstate Oxidation of Uric Acid

Flask No.	Uric acid.	5 per cent sodium cyanide.	Colorimeter reading.	Value found.
	mg.	*cc.*	*mm.*	*mg.*
1	0.25	None.	30.0	1.00 × 0.25
2	0.25	0.05	25.5	1.18 × 0.25
3	0.25	0.25	21.1	1.42 × 0.25
4	0.25	0.50	20.4	1.47 × 0.25
5	0.25	1.00	19.2	1.56 × 0.25
6	0.25	2.00	16.1	1.86 × 0.25
7	0.25	4.00	14.1	2.13 × 0.25
8	0.25	8.00	12.5	2.40 × 0.25
9	0.25	12.00	12.2	2.46 × 0.25
10	None.	10.00	No color.	None.

Color developed in each case by addition of 1 cc. of phosphotungstic acid reagent and 10 cc. of 20 per cent sodium carbonate. All made up to 50 cc. volume after 10 minutes and compared with No. 1.

TABLE II.

Effect of Sodium Cyanide upon Arsenotungstate Oxidation of Uric Acid.

Flask No.	Uric acid.	10 per cent sodium cyanide.	Colorimeter reading.	Value found.
	mg.	*cc.*	*mm.*	*mg.*
1	0.4	1.0	148.0	0.054
2	0.4	1.5	52.0	0.156
3	0.4	2.0	34.4	0.236
4	0.4	2.5	30.0	0.266
5	0.4	3.0	27.1	0.287
6	0.4	3.5	25.9	0.309
7	0.4	4.0	24.7	0.324
8	0.4	4.5	23.5	0.340
9	0.4	5.0	21.3	0 376
10	0.4	7.5	20.2	0 396
11*	0.4	10.0	20.0	0 400
12	0.4	15.0	20.0	0 400
13	None.	10.0	No color.	None.

Color developed by addition of 2 cc. of arsenotungstic uric acid reagent and the amount of cyanide indicated for each flask.

* No. 11 was used as a standard and the others compared with it.

Flask 2, due to 1 drop of cyanide, recalls an increase of the same amount which Benedict and Hitchcock observed under similar conditions (2 drops of cyanide and 15 cc. of carbonate in a 50 cc. flask). Flasks 8 and 9, with 50 per cent more cyanide in the latter, showed nearly a constant value with two and a half times as much color as Flask 1. Obviously the cyanide causes displacement of the equilibrium so as to approach the maximum amount of the blue compound for these conditions. Attempts to determine the full extent of this effect met with little success at first for the decrease of carbonate and increase of cyanide was required to accomplish further deepening of the color, and this change in the alkalies caused precipitation before the time for development of color had passed. Also we found that a certain amount of carbonate was necessary to prevent the blue color which otherwise develops when phosphotungstic acid reagent is made alkaline with sodium cyanide.

We had previously made many conjugated tungstic acids, substituting analogous acids for phosphoric, concerning which we expect to make a more extended report soon. Upon trial we found that one of these, arseno-18-tungstic acid, in addition to other desirable properties, gave absolutely no color with sodium cyanide even when no other alkali than the cyanide was present, and did not precipitate in the presence of large amounts of cyanide. By its use we made further observations of the effect of cyanide upon the oxidation of uric acid by tungsten compounds and secured the results recorded in Table II. It will be noted that the amount of uric acid used in each flask was 0.4 mg., for our experience had shown that the color obtained from that quantity was of a desirable depth for colorimetric comparisons. It is also apparent that the color in Flasks 1 to 10 increased with additional quantities of cyanide, the increments being progressively smaller. In Flasks 11 and 12 there was practically no increment, though the quantities of cyanide were, respectively, 133 and 200 per cent of that in Flask 10. Evidently the conditions had been reached by which the oxidation of uric acid by a tungsten compound was complete.

The determination of relations between the amount of color obtainable by the new arsenotungstate-cyanide method and the former phosphotungstate-carbonate procedures could not be abso-

lute, owing to the great difference in the concentrations of the reaction liquids. Approximate comparisons show that the new uric acid method gives 3.3 times the color of the Folin-Macallum-Denis method, 2.8 times that of the Benedict-Hitchcock procedure, and 2.5 times that of the Folin-Wu method. There is, of course, a great mechanical advantage in being able to get three times as much color from the very limited amount of uric acid in blood and other body fluids. In addition, there is greater satisfaction in the realization that the deeper color made possible by the new method represents a truer value of the uric acid present in that it is the result of a complete chemical action and not subject to the disturbing variations which may occur in procedures which depend upon artificially maintained equilibria.

Finally, the use of sodium cyanide in the manner described in this paper possesses another advantage in the selective application of its driving power. How exclusive this selective action of cyanide, whether there are other substances present in body fluids upon which it will act, and what the nature of the driving power may be are questions now being investigated further in this laboratory.

Method.

Reagents.

Preparation of Arseno-18-tungstic Acid Solution.—Boil a mixture of 100 gm. of hydrated sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), 125 gm. of arsenic acid anhydride (As_2O_5), and 650 cc. of water for 2 to 4 hours in a flask. If the reagent so formed has a blue or green color after it has boiled the required time, it should be decolorized by boiling with sufficient bromine water to make the color a clear yellow or yellowish brown.[10] After boiling off any excess bromine add distilled water to make the volume 1 liter. The arsenotungstic acid reagent so prepared is a somewhat lighter color than the phosphotungstic acid reagent.

Other Reagents Required.—2.5 per cent zinc chloride solution; 10 per cent sodium carbonate solution (if monohydrated sodium carbonate is used, allowance must be made for the water of crystallization); 10 per cent hydrochloric acid solution; 10 per cent sodium cyanide solution; standard uric acid solution (phosphate solution of Benedict-Hitchcock).[7]

For Removal of Proteins.[6]—10 per cent sodium tungstate; $\frac{2}{3}$ N sulfuric acid, within 5 per cent by titration; solid potassium oxalate.

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[10] Decolorization in this way is desirable for any conjugated tungstic acid which is to be used for colorimetric work. A dark blue or green reagent (either phospho- or arsenotungstic acid) introduces a very noticeable error when used where the color to be read is light.

Determination.

The method is essentially the same when used in uric acid solutions of such different concentration as urine and blood. Convenient quantities of reagents and choice of volumetric flasks which facilitate colorimetric comparison are the principal points of difference in the procedures described.

Procedure as Used in Urine.—Pipette 1 cc. of urine into a 50 cc. centrifuge tube and dilute with distilled water to about 40 cc. Add 1 cc. of 2.5 per cent zinc chloride and mix with a stirring rod. Add 1.0 cc. of 10 per cent sodium carbonate which should make the solution alkaline to litmus and stir thoroughly. Centrifuge for about 2 minutes, drain off, and discard the supernatant liquid. Dissolve the residue, with stirring, in 3 or 4 drops of 10 per cent hydrochloric acid, dilute with 5 cc. of water, add 10 cc. of 10 per cent sodium cyanide, and transfer quantitatively to a 100 cc. volumetric flask, and dilute to about 60 cc. If 1 cc. of urine contains more than 0.5 mg. of uric acid the amount of cyanide should be doubled (20 cc.) and a 200 cc. flask used. In this case dilute to about 120 cc. To prepare a standard containing 0.2 mg. in 50 cc. pipette 1 cc. of the phosphate standard solution into a 50 cc. volumetric flask and 25 to 30 cc. of distilled water and 5 cc. of 10 per cent sodium cyanide. Develop the color in both by addition of the arseno-18-tungstic acid reagent, 1 cc. to the standard (50 cc. flask), 2 cc. to the unknown if in 100 cc. flask or 4 cc. if in the 200 cc. flask. Shake, dilute to volume, let stand 2 or 3 minutes, and compare in the colorimeter. The color develops with such rapidity that the time interval indicated is sufficient if the standard and the unknown are made simultaneously. If, for any reason, they are not so prepared, it is best to allow 10 minutes to elapse before making the color comparison.

Procedure as Used in Blood.—Collect oxalated blood in the usual manner, drawing the blood from a vein into a weighed flask containing 2 mg. of potassium oxalate for each cubic centimeter of the sample taken. After determining the amount of blood by weight, pour it into seven times its volume of distilled water, add 1 volume of 10 per cent sodium tungstate solution and then, while shaking, run in slowly 1 volume of $\frac{2}{3}$ N sulfuric acid. Shake for several minutes and filter (precipitation method of Folin and Wu[6]). Pipette 25 cc. of the clear filtrate (corresponding to 2.5 cc. of blood) into a 50 cc. centrifuge tube and dilute with distilled water to about 40 cc. Add 1 cc. of 2.5 per cent zinc chloride and mix with a stirring rod. Add 1.0 cc. of 10 per cent sodium carbonate to make just alkaline to litmus and stir thoroughly. Centrifuge for about 2 minutes, drain off and discard the supernatant liquid. Dissolve the residue with stirring in 3 or 4 drops of 10 per cent hydrochloric acid, dilute with 5 cc. of water, and add 2.5 cc. of 10 per cent sodium cyanide and transfer quantitatively to a 25 cc. volumetric flask. Prepare two standards containing 0.1 and 0.2 mg. in 50 cc., by pipetting 0.5 and 1 cc. of the phosphate standard solution into two 50 cc. volumetric flasks. Add about 30 cc. of distilled

water and 5 cc. of 10 per cent sodium cyanide to each. Develop the color by the addition of the arseno-18-tungstic acid reagent, 0.5 and 1 cc. respectively into the unknown and standards. If the color has been developed simultaneously, shake, dilute to volume, let stand a minute or two, and compare in the colorimeter; if not, the same lapse of time should be allowed as indicated in the case of urine.

Both procedures are adapted to the quantities of uric acid found in the largest number of urine and blood samples analyzed by us. In a very few cases we found it advantageous to choose volumetric flasks of a larger or smaller size to contain the unknown. This may be done with good results if the concentrations of arseno-18-tungstic acid and sodium cyanide are kept comparable. For this purpose the following simple rule must be observed:

100 cc. flask contains 10 cc. of 10 per cent sodium cyanide and 2 cc. of arseno-18-tungstic acid reagent; 50 cc. flask contains 5 cc. of 10 per cent sodium cyanide and 1 cc. of arseno-18-tungstic acid reagent; 25 cc. flask contains 2.5 cc. of 10 per cent sodium cyanide and 0.5 cc. of arseno-18-tungstic acid reagent.

TABLE III.

Comparative Estimations of Uric Acid in Urine.

Urine.	Benedict-Hitchcock method.	Folin-Wu method.	New method.
	mg.	*mg.*	*mg.*
1	390	375	388
2	752	625	702
3	514	492	533
4	547	498	508

Table III presents comparative uric acid results for several urine specimens. In their analyses we used the method described here, the Folin-Wu method, and the Benedict-Hitchcock procedure. Precaution to use only the clearest possible, silver reagents had the effect of practically eliminating irregularities due to reduced silver. In spite of similar precautions in work with blood specimens there were marked irregularities. Upon undertaking the work of their explanation we were led into various problems connected with the chemistry of the methods and the chemical nature of the uric acid present in blood. Some of the results obtained appear in the following paper.[11]

[11] Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, 1922, l, 65.

SUMMARY.

Combination of zinc precipitation with a new colorimetric method has made possible the estimation of very small quantities of uric acid. Arseno-18-tungstate proves a great improvement over phospho-18-tungstate of earlier methods. Sodium cyanide is used as the only alkali for development of color and serves to bring about the complete oxidation of uric acid. In comparison with the amount of color obtained in the methods which depend upon oxidation to a point of equilibrium, the new conditions of complete oxidation permit the development of three times as much color per unit weight of uric acid. The same conditions that bring about completion of the reaction are also responsible for greater speed in reaching the maximum color and in a very marked permanency of the color. The use of cyanide as alkali practically eliminates the precipitation of various compounds in the colored liquid which, next to fading, was the most serious difficulty accompanying the use of carbonate.

Precipitation of uric acid with zinc salts lends itself just as well to the subsequent formation of a double radical with cyanide as in the case with silver methods. In addition there is excluded all possibility of a reduced metal giving erroneous results in the later oxidation reaction.

Finally, the number of reagents required is small, they are easily prepared and the use of inexpensive zinc chloride instead of expensive silver salts makes the determination much more desirable, especially where many analyses are run, as in medical classes and extended research work on purines.

STUDIES ON THE URIC ACID OF HUMAN BLOOD.

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Uric acid estimations have probably been subject to more irregularities and losses than attend the analysis of other well known biological products. Explanations of these irregularities have usually been based upon its very slight solubility, the unusual ease and variety of its oxidation reactions or the more vague property of different forms in which it has been supposed to exist in body fluids. The new method of uric acid analysis described by the authors in the foregoing paper,[1] while characterized by extraordinary agreement between successive determinations in urine and blood, differed in quite an irregular manner when its values for blood were compared with those obtained by the method of Folin and Wu.[2] The present paper sets forth some of the results of our efforts to find an explanation for the apparent discrepancies. In addition to evidence which casts considerable doubt upon the accuracy of uric acid data obtained through the use of earlier methods, we have made observations which can be explained only by the existence of uric acid in more than one form in human blood.

Differences in form of uric acid, or more correctly urates, have been used as a basis for several hypotheses concerned with the physiology and pathology of purine metabolism. One of the more definite ideas of these differences is that which pictures uric acid present in various stages of change according to the effect of blood conditions upon its property of keto-enol isomerism. Gudzent[3] first discovered that uric acid forms two series of primary

[1] Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, 1922, l, 55.

[2] Folin, O. and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

[3] Gudzent, G., *Z. physiol. Chem.*, 1909, lx, 38.

urates, differing in stability and solubility. They were named the lactam and lactim forms. Shifting the point of equilibrium between these tautomeric forms might conceivably take place under changing blood conditions. The resulting change of point of saturation has been urged as an explanation of many clinical symptoms, such as the deposition of urates in cartilages of gout patients, etc.

Another idea of the difference in form of uric acid is that some part of the whole amount is combined with another substance or substances. Minkowski[4] advanced the hypothesis that uric acid may be combined with nucleic acid in the body tissues and suggested that this combined uric acid might regulate the chemical relationships of free uric acid. The basis for this conclusion was the observation that the precipitation of a uric acid solution by acetic acid or ammoniacal silver magnesium solution is prevented by the addition of nucleic acid. No experimental evidence has been advanced that combined uric acid exists in any part of the human body. Benedict[5] several years ago found combined uric acid in mixed beef blood, in quantity many times greater than the free uric acid in the same blood. A year later Benedict[6] made the following statement, "with the exception of man, all mammals probably have two forms of uric acid in the blood. In the case of human blood the data so far available are not conclusive. It is quite probable that here, too, uric acid exists in the blood in at least two forms but they are quite unlike the forms present in ox blood." Davis and Benedict[7] recently reported the isolation of a crystalline substance from beef blood which they identified as a ribose-uric acid compound. The data presented later in this paper furnish the first positive experimental proof that human blood contains uric acid in at least two forms.

We analyzed many samples of blood and serum by the Folin-Wu method and by the new method in order to compare the values

[4] Minkowski, O., Die Gicht, in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1903, vii, pt. 2, 189–190.

[5] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 633.

[6] Benedict, S. R., *J. Lab. and Clin. Med.*, 1916–17, ii, 1.

[7] Davis, A. R., and Benedict, S. R., *J. Biol. Chem.*, 1921, xlvi, p. v.

so obtained. In all bloods the Folin-Wu method of protein precipitation was used, the filtrate in each case serving as a common source of the samples taken for both methods of analysis. Recognizing the danger of abnormal results in the Folin-Wu method due to the possible presence of "reduced silver" every precaution was taken to assure the use of clear silver lactate solution.

TABLE I.

Uric Acid Estimation in Blood. Comparison of Methods.

Specimen.	Folin-Wu method.	New method.
Group 1. Examples of blood and serum which show marked irregularities.		
	mg.	*mg.*
Blood 1, C. B. I.	4.1	4.9
" 2, G. F.	2.7	3.4
" 3, A. M.	1.8	2.3
" 4, C. M.	4.4	5.5
" 5, J. K.	1.5	2.3
" 6, J. D. III.	1.7	2.7
" 7, C. B. II.	2.7	3.4
" 8, G. C.	2.7	4.8
" 9, J. Dh. II.	1.9	3.0
Mixed serum A.	1.4	3.1
" " B.	1.6	2.5
Group 2. Examples of blood which show little or no irregularity.		
Blood 1, G. M.	7.7	7.9
" 2, J. W. II.	3.5	3.2
" 3, J. H.	3.4	3.4
" 4, I. C.	1.8	1.6
" 5, C. B. II.	3.2	3.2
" 6, D. H.	2.3	2.3
" 7, J. D. I.	1.8	2.1

In addition, suspicious variations between duplicates, were immediately followed by check determinations. In this way the Folin-Wu values which are recorded in this paper represent the highest accuracy which can be obtained by the method. Even after these unusual precautions there were puzzling irregularities in the agreement of the two series of results. Typical examples of the comparative blood analyses are presented in Table I.

It is apparent from the figures tabulated that the new method gives values for blood which range from those which are essentially the same (Group 2, Bloods 1 to 7) to those (Group 1, Bloods 1 to 9) which are higher than the corresponding Folin-Wu values. The largest increase in value shown in the bloods is 75 per cent. In the case of the mixed serums, A and B, the results are similar but the amounts of increase range from 60 to 120 per cent. This high range of increase is characteristic of all mixed serums we have had under observation. We made repeated attempts to lower the results of the new method to the level of the Folin-Wu method. We could neither accomplish such a decrease nor find evidence of the presence of any substance which gave an added color value. The specific nature of the new cyanide-arsenotungstic reaction (in its driving effect upon uric acid to the exclusion of any other substance so far investigated) was good evidence that the higher value is due to a more complete measurement of the uric acid present. Attempts were then made to increase the Folin-Wu values. None of these was successful until we added potassium oxalate. Though smaller amounts of oxalate have the effect of elevating the value obtained with the Folin-Wu method, we found it desirable to use an excess sufficient for all possible variations in blood specimens. Therefore, we added 100 mg. of potassium oxalate (measured as 10 cc. of a 1 per cent solution) to each 20 cc. quantity of filtrate before beginning the determination. The presence of the oxalate increased the results of the determination as shown in Table II.

The recorded results obviously fall again into two groups. The values of the Folin-Wu determinations are markedly increased in the first group of seven blood samples and two mixed serums while there is no increase or a very slight increase in the second group of six bloods. Evidently the value found by the new method determines not only the question of whether an increase will result from the use of potassium oxalate but constitutes the approximate limit of the increase when present. With minor differences of the order of variation between duplicates, the figures secured by means of the modified Folin-Wu method (potassium oxalate preceding the Folin-Wu procedure) are the same as those obtained by the new method. This observation is peculiarly significant when considered in connection with the

fact that the presence of oxalate had little or no effect upon the analysis of those blood specimens (entered as Nos. 1 to 6 of Group 2, Table II) for which both methods gave essentially the same values. The explanation of the irregularities was thus shown to be less a matter of method weakness and more a matter of character of the content of individual bloods.

TABLE II.

Effect of Potassium Oxalate upon Blood Uric Acid Values Obtained by the Folin-Wu Method.

Specimen.	Folin-Wu method.	Folin-Wu method after addition of $K_2C_2O_4$.	New method.
Group 1. Examples of blood and serum which show a marked increase.			
	mg.	*mg.*	*mg.*
Blood 1, C. B. I.	4.1	5.2	4.9
" 2, G. F.	2.7	3.5	3.4
" 3, A. M.	1.8	2.3	2.3
" 4, C. M.	4.4	5.4	5.5
" 5, J. K.	1.5	2.7	2.3
" 6, J. D. III	1.7	2.2	2.7
" 7, C. B. II	2.7	3.2	3.4
Mixed serum A.	1.4	4.3	3.2
" " B.	1.6	3.3	2.5
Group 2. Examples of blood which show little or no increase.			
Blood 1, G. M.	7.7	8.0	7.9
" 2, J. W. II	3.2	3.3	3.2
" 3, J. H.	3.4	3.7	3.4
" 4, I. C.	1.8	1.6	1.6
" 5, C. B. II	3.2	3.6	3.2
" 6, D. H.	2.4	2.3	2.2

We undertook an investigation of different blood specimens in the hope of finding what chemical difference exists that determines for each specimen whether the values given by the silver and zinc methods are to agree or disagree and a corresponding agreement or disagreement between the values by the former method in its original form and as modified by the addition of oxalate. The very limited quantity of blood in each case hindered

progress of the investigation as did also the impossibility of judging before analysis whether each new specimen would show agreement or disagreement between the methods. The use of other than human blood was inadvisable in view of the radical difference in form and quantity recognized by Benedict as characterizing the blood uric acid of different species. Analysis of mixed serums (from many blood specimens drawn for routine Wassermann tests and found negative) consistently showed the new method value higher than the Folin-Wu value. Furthermore, the percentage increase was about twice as great as in the case of whole blood specimens. Evidently serum was by nature and quantity availability the best material for use in identifying the character of the substance responsible for the divergent uric acid values. Serum was saved over a period of weeks. The proteins were precipitated from each day's quantity of serum by the tungstic acid method. The filtrates of successive days were poured together until there was a volume of 5 liters. Several such lots were investigated after each was analyzed by the three procedures. The two sets of results presented in the foregoing tables are typical analyses of mixed serum.

As a result of many observations on the mixed serums we were convinced that we were dealing with more than one form of uric acid. We attempted the separation of uric acid as such for evidence supporting the analytical data. 1,000 cc. of mixed serum were precipitated in 50 cc. portions by the zinc procedure. After centrifuging and pouring off the mother liquid the combined precipitates were dissolved in 10 per cent acetic acid, then 100 cc. of water and about 0.5 gm. of bismuth carbonate added. Hydrogen sulfide was bubbled through the solution until it was saturated; it was then heated to the boiling point and filtered. The filtrate was evaporated to small quantity and then heated to dryness on the water bath. After dissolving in 25 cc. of hot water and transferring to a 50 cc. centrifuge tube, the uric acid was again precipitated by the addition of 4 cc. of the ammoniacal silver magnesium reagent of Benedict and Hitchcock and separated by centrifuging.[8] To the precipitate were added 5 cc.

[8] The second precipitation (as a silver compound) and subsequent separation of uric acid as such substantially follows the method used by Benedict in identifying uric acid in beef blood (Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 637).

of water and 5 cc. of saturated hydrogen sulfide solution (freshly prepared). After mixing thoroughly the silver sulfide was removed by filtration and the hydrogen sulfide from the filtrate by evaporation to dryness on a water bath. The residue was taken up in successive small portions of boiling water and transferred to a weighed 15 cc. centrifuge tube. The volume was then about 5 cc. 1 cc. of glacial acetic acid was added and the tube set aside. 5 days later the mother liquor was poured off and the characteristic crystalline precipitate of uric acid washed first with water and then with alcohol. After drying, the tube was weighed. In addition to the weight and crystalline form further evidence of the nature of the precipitate was obtained by colorimetric analysis. The characteristic acid precipitation was checked by the data so secured. The results are presented in Table III and with them are recorded, for comparison, the values obtained in the similar treatment of a standard uric acid solution, which contained 3 mg. and was diluted to a liter before precipitation.

Reference to the table shows in the case of each serum that, after the laborious process of removing uric acid from solution twice by formation of two different salts, the amount found present by analysis of the final crystalline product is greater than the Folin-Wu method originally indicated. (Serum A shows 2.2 mg. against 1.4 mg. and Serum B shows 2.0 mg. against 1.6 mg.) On the other hand, the amount of uric acid which could be similarly recovered from the standard solution is much less than was originally present (1.2 mg. from 3.0 mg.). The latter yield is as large as might be expected after the tedious steps of the double precipitation, the subsequent removal of the metallic sulfides, filtrations, evaporations, etc. If the loss in the case of the serum filtrates was actually comparable to that of the uric acid solution, and we should not expect it to be otherwise, the original amount of uric acid in the mixed serums must have been about 4 mg. Analysis by the new method and the Folin-Wu method after the addition of oxalate gave values of 3.2 and 4.3 mg. for Serum A, 3.2 and 3.5 mg. for Serum B. Similar treatment of other mixed serums resulted in a yield of separated uric acid which invariably exceeded the Folin-Wu figure. Repeated attempts to obtain larger amounts of uric acid from standard solutions containing 3 mg. in a liter, never resulted in a yield higher than 50 per cent.

It cannot be supposed that the significance of these facts is only a demonstration that the Folin-Wu method gives low results. Such bloods as those of Group 2, Tables I and II, disprove such an idea. There the range of uric acid amount is from 1.6 to 7.9 mg. and the two methods agree. The explanation can only be that

TABLE III.

Identification of Uric Acid Removed as Such from Blood Serum and Uric Acid Solution.

		Uric acid content of solution.	Colorimetric analysis of uric acid separated.	Weight of crystalline precipitate.
		mg.	*mg.*	*mg.*
Serum A.	Folin-Wu..........	1.4	Twice washed crystals..........2.2	
	Folin-Wu following oxalate.....	4.3	Mother liquor.....0.0	3.1
	New method......	3.2	Wash water and alcohol..........0.02	
Serum B.	Folin-Wu.........	1.6	Twice washed crystals..........2.0	
	Folin-Wu following oxalate......	3.5	Mother liquor.....0.33	2.2
	New method......	3.2	Wash water and alcohol..........0.07	
Uric acid standard..........		3.0	Twice washed crystals..........1.2 Mother liquor.....0.23 Wash water and alcohol..........0.02	2.2

uric acid is present in the mixed serums and some bloods (Group 1, Tables I and II) in more than one form. In other bloods (Group 2, Tables I and II) there is but one form, or traces only of the second form. In consideration of the fact that the Folin-Wu method and the new method give quite comparable results when

applied to standard uric acid solutions, it must be the second form of uric acid which the Folin-Wu method fails to include while the new method includes it. That it is some form of uric acid rather than any other substance which reacts colorimetrically follows of necessity from the facts here presented that: (*a*) it carries successively through the precipitations with zinc salt and silver magnesium mixture, which are chemically different but equally characteristic; (*b*) it then precipitates quantitatively upon acidification of its solution in the form of crystals which cannot be differentiated from those of uric acid; (*c*) it is changed quantitatively at room temperature in contact with potassium oxalate to a form readily precipitated and extracted by the usual Folin-Wu procedure; and (*d*) the new method gives a value for this second form, as well as the first, in spite of the exclusion of all substances so far tried from the multiplying effect of the cyanide upon the color. Further work to determine the chemical nature of the second form of uric acid is now under way in this laboratory.

As observed above; there is apparently a greater relative amount of the second form of uric acid in serum than in whole blood. We secured freshly drawn samples of blood of sufficiently large volume to allow three sets of analyses by the original Folin-Wu method, Folin-Wu following oxalate method, and the new method. Three determinations were run upon a filtrate from the whole blood, three more upon a filtrate from a serum portion, and another three upon a filtrate from a corpuscle portion. Approximate separation of the bloods was effected by means of the centrifuge. While analysis of the serum portion and corpuscle portion does not furnish strictly quantitative data on the uric acid content of either serum or corpuscles uncontaminated by the presence of small amounts of the other, nevertheless the results unmistakably indicate the order of uric acid distribution between the corpuscles and serum. The figures of Table IV, which represent that distribution in a typical blood specimen would, by more complete separation of the formed elements, be changed so as to further emphasize the fact that the uric acid content of serum is from one and a half to nearly twice that of the corpuscles. Values by the Folin-Wu following oxalate method are 5.7 mg. against 3.0 mg. and the corresponding new method figures are 4.1 mg.

against 2.3 mg. The results obtained when using the original Folin-Wu method are to be considered very approximate since the color due to the small amount of uric acid present was too small for accurate estimation. The second form uric acid, apparent in the table as the difference between the new method and the Folin-Wu method values, is present in the serum portion to an extent three times as great as the first form, 3.2 mg. against 0.9 mg. and in the corpuscle portion is twice as great, 1.5 mg. against 0.8 mg. Such direct observations of the relatively smaller second form uric acid content of corpuscles substantiate the relatively large second form uric acid content of serum previously mentioned. Also we have interpreted these observations, that the added uric acid value is unevenly distributed between corpuscles and serum, as further evidence supporting the existence of the second form of blood uric acid.

TABLE IV.

Distribution of Uric Acid in Blood.

	Folin-Wu method.		Folin-Wu method following $K_2C_2O_4$.		New method.	
Whole blood	2.2		3.9		3 6	
Serum portion	0.9	0.4	5.7	3.0	4.1	2.2
Corpuscle portion	0.8	0.3	3.0	1.4	2.3	1.3

The figures recorded in the first column for each method represent quantities in 100 cc. of serum or corpuscles.

The figures in the second column represent quantities in 100 cc. of blood.

Finally, it should be noted that the distribution of uric acid just described for human blood is in marked contrast with that observed by Benedict[5] for mixed ox blood. In the latter Benedict found all the uric acid (free and combined) in the corpuscles, none in the serum. In the former we find the uric acid in both, but in much greater quantities in the serum.

This different distribution suggests that the second form of uric acid in human blood is probably different from the "combined" uric acid of ox blood. Whether this is the case is only one of the many important questions, which we hope may be attacked by means of the new zinc precipitation-arsenotungstate cyanide

method. We are already studying some of these problems and expect to investigate others as rapidly as new facts concerning the forms of uric acid can be ascertained. Until there is thus developed a more complete understanding of blood uric acid of different species it probably is not desirable to theorize on the physiological and pathological significance of the second form of uric acid in human blood.

Our thanks are due to Dr. H. W. Gauchat of the Cleveland City Hospital for enthusiastic cooperation in securing suitable blood specimens for the particular requirements of this investigation. We gratefully acknowledge the technical assistance rendered by Mr. H. W. Hottenstein. We are also indebted to Dr. E. E. Ecker for making available large quantities of serum from the Wassermann laboratories of Lakeside Hospital and the Cleveland Board of Health.

EXPERIMENTAL RICKETS IN RATS.

III. THE PREVENTION OF RICKETS IN RATS BY EXPOSURE TO SUNLIGHT.*

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PLATES 2 AND 3.

(Received for publication, October 21, 1921.)

In recent papers it was shown by Hess and Unger that rickets in infants could be cured by frequent short exposures to the sun's rays (1, 2). By this means and without any alteration whatsoever of the dietary, the characteristic signs of this disorder markedly diminish in 3 to 4 weeks, as noted by clinical examination and by the x-ray. As a result of favorable experiences of this nature it was concluded in a study of "the seasonal incidence of rickets" (3) that "hygienic factors, especially sunlight, and not dietetic factors, play the dominant rôle in the marked seasonal variations of this disorder." It seems probable that the ultraviolet rays play a large part in this curative power of the sun, judging from the work of Huldschinsky (4) and others (5, 6, 7) who recently have shown that infantile rickets can be cured by means of the rays produced by the mercury-vapor lamp. In 1918 we tried the curative effect of rays from this source, but, lacking the aid of x-ray examinations, could not convince ourselves of their efficacy; since then we have succeeded in curing rickets by this means.

Having found sunlight efficacious in the rickets of infants, we proceeded to test its value in the prevention of rickets in rats. To this end a series of white rats was placed on the diet (No. 84) described by Sherman and Pappenheimer (8) consisting of patent flour 95.0 per cent, calcium lactate 2.97 per cent, sodium

* Read in abstract before the Society of Experimental Biology and Medicine, October 19, 1921.

chloride 2.0 per cent, and ferric citrate 0.1 per cent. It has been the experience of the investigators in this laboratory that such a diet invariably leads to the development in rats of lesions which are anatomically identical with those of infantile rickets.

In carrying out experiments on rats our practice had been to keep the colony in a semidark room, the yellow shades being drawn at all times. In testing the effect of sunlight, the rats (weighing at the outset about 40 gm.) were kept in absolute darkness, one series being taken out of the room and exposed to the direct sunlight for a period of 15 or 30 minutes. There was no difference whatsoever in the diets of these two groups. After a period of about 3 weeks the animals were radiographed in order to observe early lesions of the epiphysis, and after 30 to 40 days were killed and autopsied. These experiments were begun in April, when the weather permitted four to five exposures a week.

It was found for the first time in our experience that Diet 84, the "rachitic dietary," did not lead to rickets—that the rats which received sun treatment did not show signs of rickets either by x-ray or by histological examination of the bones. It is unnecessary to discuss in detail the histological criteria which we consider characteristic of rickets, as this question has been fully considered in a previous paper (9). It may be stated briefly that they consist of increased width and irregularity of the proliferative cartilage, absence of calcium deposition, and great excess of osteoid in the region of the metaphysis and along the shafts of the bones. It will be seen from Figs. 1 and 3 that the rats which were kept at all times in the dark showed these lesions, whereas the bones of those exposed to the sun did not show them (Figs. 2 and 4).

In the paper previously referred to it was shown that the introduction of 0.4 per cent of secondary potassium phosphate (K_2HPO_4) in place of an equal weight (replacing about one-seventh of the calcium lactate contained in the rickets-producing diet) completely prevented the development of rachitic lesions; this constitutes an addition of 75 mg. of phosphorus per 100 gm. of the diet. In order to test the counterbalancing effect of phosphate and darkness, a series of tests was carried out in the dark with additions of small and increasing amounts of potassium

TABLE I.

Diet.	Duration.	Rat No.	X-ray.	Microscopic examination.
Darkness.				
	days			
No. 84 86 mg. P	34	246	Rickets.	Rickets.
	23	247		"
	22	248		"
72 mg. P		436	Rickets.	
		437	"	
	30	438	"	Rickets.
No. 84 + 25 mg. P	39	262	"	"
	39	263	"	"
	39	264	"	"
	28	443	"	
	28	444	"	Rickets (slight).
	28	445	"	
No. 84 + 75 mg. P	38	121	Negative.	Negative.
	38	122	"	"
	38	123	"	"
Sunlight.				
No. 84 86 mg. P	34	249	Negative.	Negative.
	32	250	"	"
	35	251	"	"
	33	439	"	"
	33	440	"	"
	33	441	"	"
	33	442	"	"
No. 84 + 25 mg. P	39	259	"	"
	39	260	"	"
	39	261	"	"
No. 84 + 75 mg. P	38	124	"	"
	38	125	"	"

phosphate to the standard dietary (No. 84); to one series 25 mg. were added, to another 75 mg. (constituting Dietary 85).

The rats on these diets were kept in the dark but, to serve as control, half of each series was exposed to sunlight for 30 minutes daily when this was possible. As was to be expected in view of our previous experience and the fact that phosphate tends to protect against rickets, none of the rats which were treated with sunlight developed rachitic lesions. Among the group, however, which was kept at all times in the dark, active rickets developed in spite of an addition of 25 mg. of phosphorus. The addition of 75 mg. was found to be sufficient to prevent the development of this disorder. This amount constituted the minimum protective supplement to Diet 84, which in itself contains about 86 mg. of phosphorus. Thus it will be noted that a short exposure to sunlight was equivalent to almost doubling the protective dose of phosphate. If the phosphate content of the diet is adequate, rats do not develop rickets in spite of being kept in the dark throughout the experiment.

The effect of sunlight with other dietaries was also studied, and is being continued. Without entering at this time into a detailed discussion of their influence, it may be of interest to record the observation that in one series of animals where 10 per cent of egg albumin was substituted for an equivalent amount of flour, rickets developed in some of the rats in spite of the sunlight treatment; whether this is to be attributed to a reduction of phosphate incidental to diminishing the percentage of flour, or to the injurious effect of the egg albumin itself, will be determined by experiments which are in progress. Possibly the increased rate of growth of these animals with the accompanying increased phosphorus requirement may have been a factor of moment.

DISCUSSION.

As sunlight has a marked effect on the bony development of rats, it is evident that in future in similar nutritional investigations, the light factor will have to be controlled and standardized. It seems probable that some of the irregularities and lack of conformity observed by investigators in this field may be attributed to keeping the experimental animals under dissimilar intensities

of light. The most interesting aspect of the question, however, is the phenomenon that the sun's rays are able to stimulate a deposition of inorganic salts where these are lacking. The damaging effect of darkness emphasizes the fact that sunlight is of great importance, not merely for the vegetable world but also for the higher animals. Furthermore, the fact that sunlight is efficacious in the rickets of both human beings and rats, serves to show the similarity of this disorder in these two species. These results indicate that in the prevention and causation of rickets at least one hygienic factor plays an important rôle which will have to be carefully considered in future studies of this disorder.

CONCLUSIONS.

Rachitic lesions which develop regularly in rats upon a diet adequate in calcium but low in phosphorus, may be prevented by short exposures to direct sunlight.

This protection is equivalent to the addition of at least 75 mg. of phosphorus to the diet in the form of basic potassium phosphate.

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EXPLANATION OF PLATES.

Plate 2.

Fig. 1. Rat 246. 34 days on Diet 84. Darkness. Chondrocostal junction showing advanced rickets. (Silver nitrate—Van Gieson stain.)

Fig. 2. Rat 249. Same litter as Rat 246. 34 days on Diet 84. Sunlight. Chondrocostal junction showing no rickets. (Silver nitrate—Van Gieson stain.)

Plate 3.

Fig. 3. Rat 263. 39 days on Diet 84 plus 25 mg. of P added as K_2HPO_4. Darkness. Radiograph showing rachitic changes at knee-joint.

Fig. 4. Rat 261. Same litter as Rat 263. 39 days on identical diet. Sunlight. No rickets.

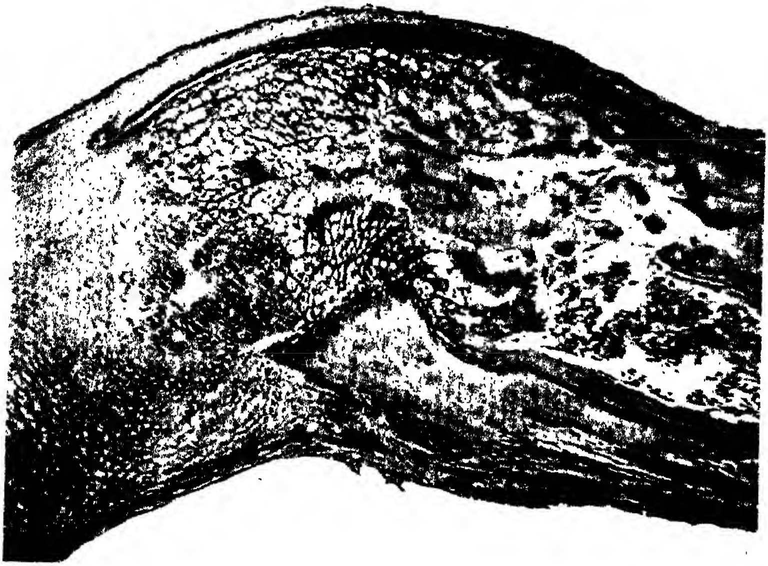

FIG. 1.

FIG. 2.

(Hess, Unger, and Pappenheimer: Experimental rickets in rats. III.)

Fig. 3.

Fig. 4.

SOME HUMAN DIGESTION EXPERIMENTS WITH RAW WHITE OF EGG.

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(Received for publication, November 16, 1921.)

The behavior of raw white of egg in the alimentary tract of the dog has been studied extensively by Bateman[1] but there does not appear to be in the literature any corresponding investigation in regard to the digestibility of white of egg in this state by the human subject. Falta[2] reports two experiments in which dried egg albumin (presumably raw) was added to a basal ration, and the amount of nitrogen in the feces determined.

TABLE I.

Experiment No.	N intake on basal ration.	With egg added.	Per cent of N from egg albumin.	Total N in feces.		Per cent of intake lost in feces.	
				On basal ration.	With egg added.	On basal ration.	With egg added.
	gm.	*gm.*		*gm.*	*gm.*		
1	16.40	27.68	40.0	1.12	1.81	7.0	6.5
2	19.20	36.12	47.0	6.05	6.25	31.0	17.3

From these figures it appears that the addition of considerable amounts of dried egg albumin (80 gm. in the first experiment and 120 gm. in the second) instead of depressing the coefficient of digestibility actually raised it when the larger amount of egg was taken, the coefficient on the basal ration in the second experiment being 69 per cent and on the egg ration 83 per cent.

Falta followed the nitrogen elimination in the urine and found that the highest point was reached somewhat later for egg albumin

[1] Bateman, W. G., *J. Biol. Chem.*, 1916, xxvi, 263.

[2] Falta, W., *Deutsch. Arch. klin. Med.*, 1906, lxviii, 517.

than for gelatin or casein. It seems clear that native egg white offers some resistance to the speedy action of the digestive enzymes, but as Bayliss[3] has shown, though trypsin acts more quickly at first on cooked egg albumin, it will if sufficient time be allowed digest the uncooked as completely as the cooked.

Wolf and Österberg[4] studying primarily the urinary nitrogen and sulfur on diets in which various protein foods were in turn added to a simple mixed ration, determined nitrogen in food and feces and found in one case with a total intake of 23 gm. of nitrogen 70 per cent of which was derived from raw egg white, the loss of nitrogen in the feces was 41 per cent of the intake; but in another period, with total intake of 14 gm. and 51 per cent of the nitrogen from the egg white, the loss was only 15 per cent of the total intake or about the same as on the basal ration alone. It would scarcely seem fair to draw conclusions from these two conflicting experiments.

The authors have accordingly conducted experiments on ten subjects,[5] all healthy young women, who took daily from ten to twelve whites of eggs as a part of a simple mixed diet, first cooked, in a 3 day period, then raw for the same length of time. The diet was uniform throughout the experiment and furnished 67 gm. of protein, to which the egg whites contributed 48 gm. or 70 per cent of the total. The experiments were divided into three groups, one in which the raw egg whites were taken thoroughly beaten, one in which they were taken in their natural state, and a third in which half were beaten and half unbeaten. In no case was there any sign of indigestion, such as discomfort or diarrhea, though one or two subjects found them slightly laxative. The cooked eggs were never subjected to a temperature or method of cooking (such as frying) which would render them tough or otherwise interfere with ease of digestion.

Coefficients of digestibility have been calculated for the total protein of the diet, which seems the fairest way to judge experiments of this sort; and also in the conventional way for the egg

[3] Bayliss, W. M., The nature of enzyme action, London, 1908, 148.

[4] Wolf, C. G. L., and Österberg, E., *Biochem. Z.*, 1912, xl, 234.

[5] Some of these experiments have been reported in a preliminary paper (Rose, M. S., and MacLeod, G., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 119).

protein alone, making average allowance for the loss of the protein of the other foods in the diet.

The cooked eggs were uniformly well digested, coefficients ranging from 83 to 91 per cent with an average of 86 per cent for the diet as a whole; or from 82 to 93 per cent with an average of 86 per cent for the egg white alone.

On the whole the raw whites were well utilized, the average difference between the cooked and raw being only 4 per cent for the protein of the whole ration or 5.5 per cent for the egg white protein alone, in favor of the cooked.

The differences between the cooked and the raw whites varied with the mode of preparation, those beaten light being the best utilized, and those taken in the natural state least well absorbed, as shown by Table II.

TABLE II.

Difference between Coefficients of Digestibility of Egg Whites in Favor of Cooked Whites.

Group No.	Mode of preparation.	Per cent of difference.	
		For whole ration.	For egg protein alone.
I	Unbeaten.	+6.8	+9.6
II	Half beaten and half unbeaten.	+3.3	+4.6
III	Beaten light.	+1.7	+3.0

EXPERIMENTAL.

The daily ration consisted of the same foods in all ten cases, but the proportions of the individual foods differed slightly, as indicated below.

Analyses for total nitrogen were made in the laboratory and protein calculated as N × 6.25.

The feces were marked off by carmine, and analyzed for total nitrogen in 3 day periods. The output of each subject is given in Table IV.

The coefficients of digestibility calculated from the foregoing are given in Table VI. In estimating the coefficients for the egg white alone, the following arbitrary allowances have been made for loss in digestion of the protein of the other items in the diet.

TABLE III.

Daily Intake of Food.

Food materials.	For Subjects G. S., M. K., L. S., M. F., M. R., F. R.		For Subjects G. B., E. B., M. E., D. T.	
	Weight of food.	Protein.	Weight of food.	Protein.
	gm.	*gm.*	*gm.*	*gm.*
Egg whites...........	372	47.66	375	47.63
Rice..................	85	6.80	85	8.75
Cream................	123	2.72	79	1.98
Saltines..............	28	3.05	35	4.68
Lettuce...............	85	1.49	85	1.00
Fruit juice...........	600	5.06	600	3.07
Butter................	39	0.39	39	0.39
Olive oil.............	33		33	
Sugar.................	50		50	
Total..............		67.17		67.47

TABLE IV.

Daily Output of Nitrogen in Feces Calculated to Protein.

Group No.	Subject.	Nitrogen in feces × 6.25.	
		Raw egg diet.	Cooked egg diet.
		gm.	*gm.*
I (Raw whites, unbeaten).	M. F.	11.24	16.35
	E. B.	10.00	8.13
	M. E.	15.25	8.43
II (Raw whites, half beaten, half unbeaten).	G. S.	9.59	11.70
	L. S.	9.29	11.78
	M. R.	8.19	11.56
	F. R.	11.26	12.22
III (Raw whites, beaten light).	M. K.	11.31	10.83
	G. B.	12.56	10.26
	D. T.	7.94	6.30

TABLE V.

Food material.	Allowances for loss in digestion of protein.
	per cent
Rice	17
Saltines	12
Lettuce	17
Cream	3
Butter	3
Fruit juices	15

TABLE VI.

Coefficients of Digestibility for Raw and Cooked Egg Whites.

Group.	A. For total protein of ration.				B. For protein of egg whites only.		
	Subject.	Cooked egg white.	Raw egg white.	Difference in favor of cooked egg white.	Cooked egg white.	Raw egg white.	Difference in favor of cooked egg white.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I (Raw whites, unbeaten).	M. F.	83.3	75.7	+ 7.6	81.9	71.2	+10.7
	E. B.	87.9	85.3	+ 2.7	88.7	84.8	+ 3.9
	M. E.	87.5	77.4	+10.1	88.1	73.8	+14.3
Average for Group I.		86.2	79.4	+ 6.8	86.2	76.6	+ 9.6
II (Raw whites, partly beaten and partly unbeaten).	G. S.	85.7	82.6	+ 3.1	85.4	81.0	+ 4.4
	L. S.	86.2	82.5	+ 3.7	86.0	80.8	+ 5.2
	M. R.	87.9	82.9	+ 5.0	88.8	81.2	+ 7.0
	F. R.	83.3	81.9	+ 1.4	81.8	79.8	+ 2.0
Average for Group II.		85.8	82.5	+ 3.3	85.3	80.7	+ 4.6
III (Raw whites, beaten light).	M. K.	83.2	83.9	− 0.7	81.8	71.2	− 1.0
	G. B.	84.8	81.4	+ 3.4	84.2	79.4	+ 4.8
	D. T.	90.6	88.2	+ 2.4	92.5	89.1	+ 3.4
Average for Group III.		86.2	84.5	+ 1.7	86.8	83.8	+ 3.0
Average for all cases.		86.0	82.0	+ 4.0	85.9	80.4	+ 5.5

CONCLUSIONS.

Raw whites of eggs, in as large amounts as ten to twelve whites daily, are well utilized in the human subject, the average coefficient of digestibility calculated for the raw egg white alone being 80 per cent as compared with 86 per cent for cooked whites in the same diet. The absorption varies with the method of preparation, being less for raw egg whites taken in their natural state than when beaten light. A mixture of whites partly beaten and partly unbeaten gave an intermediate value. The quantities consumed are regarded as maximal in dietary practice, and it seems unnecessary to emphasize the difference between raw and cooked eggs if the raw eggs are beaten.

A MODIFICATION OF FOLIN'S COLORIMETRIC METHOD FOR THE DETERMINATION OF URIC ACID.*

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(From the Chemical Division, Medical Clinic, the Johns Hopkins University and Hospital, Baltimore.)

(Received for publication, October 19, 1921.)

INTRODUCTION.

In 1912, Folin and Denis (1) introduced phosphotungstic acid as a color reagent for the detection of uric acid, and from Folin's laboratory, within a year, appeared methods for the quantitative estimation of uric acid in urine (2, 3) and blood (4). The method was greatly improved by Benedict and Hitchcock's (5) discovery that cyanide very considerably increased the intensity of the color developed and retarded the fading, and recently, the method[1] has been still further improved by Folin and Wu (6). Volumetric methods have been proposed by Curtman and Lehrman (7) and Morris (8), but the colorimetric procedure has been the method most commonly used.

* A preliminary report of this work appears in *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 126.

[1] Transfer a measured volume of blood into a flask having a capacity of fifteen to twenty times that of the volume taken. Dilute the blood with 7 volumes of water and mix. With an appropriate pipette, add 1 volume of 10 per cent solution of sodium tungstate ($Na_2WO_4 2H_2O$), and mix.

With another pipette, add to the contents of the flask (with shaking), 1 volume of $\frac{2}{3}$ normal sulfuric acid. Close the mouth of the flask with a rubber stopper and give a few vigorous shakes. Much oxalate or citrate interferes with the coagulation and later with the uric acid determination. 20 mg. of potassium oxalate are ample for 10 cc. of blood. When the blood is properly coagulated, the color of the coagulum changes from pink to dark brown. If this change does not occur, the coagulation is incomplete, but the sample may be saved by adding 2 N sulfuric acid, drop by drop, shaking vigorously after each addition, and allowing the mixture to stand for a few minutes before adding more, until the coagulation is complete. Pour the mixture on a filter paper large enough to hold the entire contents of the flask and cover with a watch-glass. The filtrate should be water-clear.

The method is simple and accurate and is admirably adapted to the tungstic acid protein-free filtrate so widely used in blood analysis.

There are, however, two disadvantages which in routine practice are of some importance; first, the relatively slight intensity of the color developed with such amounts of uric acid as are in normal blood; and secondly, the troublesome crystalline precipitate which often appears in the colored solution, thereby rendering reading impossible without filtration, a procedure which somewhat diminishes the color. We have endeavored to overcome these difficulties and believe we have succeeded.

In an effort to find the cause for the troublesome precipitate developing in the colored solution, we found that if Folin's uric acid reagent were dialyzed in heavy parchment membranes against large amounts of tap water, until all the free acid was gone, and the solution so dialyzed was evaporated to dryness, a reagent was obtained which, in the presence of uric acid and an excess of NaCN, gave a very intense color and a more or less dense flocculent precipitate. The latter did not alter in amount or character over a period of 24 hours or more. If, on the other hand, Folin's uric acid reagent were boiled cautiously to dryness without dialysis, a reagent was obtained, which, in the presence of uric acid and an excess of sodium cyanide gave the same intense color, but also a dense crystalline precipitate in the course of 3 to 5 minutes. This last reagent, which we call sodium phosphotungstate "B" when mixed with the dialyzed sodium phosphotungstate "D" will cause dissolution of the flocculent precipitate and at the same time, no crystalline precipitate will develop unless too much "B" is added. The proper mixture of these two salts results in a reagent which under conditions of the determination, gives a color nearly five times as intense as that given by Folin's procedure and no precipitate results.

Preparation of Phosphotungstic Reagents.

Preparation of Phosphotungstate "B".—1,000 cc. of Folin's reagent[2] in a large porcelain casserole are boiled rapidly over a

[2] Sodium tungstate (100 gm.), 85 per cent phosphoric acid (80 cc.), plus water (700 cc.) boiled with reflux condenser for 24 hours. Cooled and diluted to 1 liter.

free flame until the volume is about 400 cc. The size of the flame is then reduced and boiling gently takes place, until the whole is the consistency of pea soup. If the solution turns green, decolorize with bromine water. At no time should the temperature of the fluid rise above 110°C. Cool in the ice box or ice solution until the solution is about 10°C. A mass of heavy yellow crystals will separate out. Allow them to settle. Pour the supernatant syrupy liquid through a Buchner suction funnel and filter the crystals off in the same funnel. Suck as dry as possible with tamping and strong suction, continuing suction for 3 to 4 hours. Then dry on filter paper over night in an incubator (37°C.). The crystals are sodium phosphotungstate "B", contaminated with a small amount of sodium phosphate. They should be perfectly dry. Yield 90 gm.

Preparation of Phosphotungstate "D".—1,000 cc. of Folin's solution are placed in a sac of "special" parchment paper, capable of holding 4 liters, and dialyzed against 10 liters of tap water for 5 days. The water should be changed once a day. Otherwise, the procedure needs no attention. The solution in the sac will increase to about 3,000 cc. At the end of 4 or 5 days, titrate 5 cc. of the solution with 0.1 N NaOH, using phenolphthalein as the indicator. 5 cc. of a 20 per cent solution of "D" neutralizes about 15 cc. of 0.10 N NaOH. To be sure that all the free phosphoric acid has gone from the dialyzed fluid, it is necessary to titrate. The total titration value of the solution should be 1,000 to 1,600 cc. If more alkali is needed, dialyze another day to get rid of the remaining phosphoric acid. If not, proceed as below.

Transfer the solution in the sac to a large casserole and evaporate over a free flame. If the solution turns green, decolorize with bromine water as often as necessary. As the amount of solution approaches 400 cc. the flame is lowered, and the boiling takes place cautiously, until the amount of solution is about 200 cc. The solution is now transferred to the steam bath, and evaporation continued until solid material begins to separate out. Do not allow the solid mass to become hard, so that it cannot be broken easily with a stirring rod. When *almost* dry, that is, when no free liquid is seen, break up into small lumps and complete the drying in the air with occasional breaking up of the

lumps and occasional heating in the steam bath. If dried *completely* without breaking up, the mass becomes stony hard and is very difficult to get out of the casserole. This salt is sodium phosphotungstate "D". Its properties will depend on the dialyzing paper used in its preparation. The heaviest grade of dialyzing paper made by the Reeve-Angel Company, 7 Spruce Street, New York City, is satisfactory. In this laboratory we have used a very heavy Belgian parchment paper, purchased before the war. It has given far better results than any other paper, but we have been unable to duplicate it. Celloidin sacs are useless, as the reagent passes rapidly through such membranes. Ordinary dialyzing papers yield only "B", or "B" and "D" mixed. Papers otherwise unsatisfactory had been made better by coating the inner surface of the paper with a thick celloidin membrane, but we have not employed this procedure sufficiently to speak with assurance as to its value. The longer the dialysis the more intense are the specific properties of "D", as distinguished from those of "B", but also the smaller the yield. 5 days dialysis is the average time necessary for 1,000 cc. of Folin's solution. Smaller quantities take a shorter time and *vice versa.* In case of doubt, it is better to dialyze another day. If any free phosphoric acid is left in the solution, the reagent is spoiled in evaporation. Yield about 85 gm.

Preparation of the Reagent Mixture of "B" and "D".

Make a 20 per cent solution of "B" water. Also make a 20 per cent watery solution of "D". Decolorize with bromine water, if not clear yellow. Boil off the excess of bromine. "B" will dissolve in water completely giving a perfectly clear solution. There is usually an insoluble residue in "D", and the solution should be warmed and filtered. If the "B" salt alone be used in analysis, a crystalline precipitate will form in the final colored solution on standing. Should the "D" salt alone be used, no *crystalline* precipitate will form, but a more or less dense *flocculent* precipitate will form immediately. When the "D" salt is pure this precipitate will remain unaltered for 24 hours or more. This flocculent precipitate undergoes dissolution giving a clear solution if some of the *"B"* be added. If too *little* "B" be added, the flocculent precipitate will not disappear; if too *much,* it will

disappear rapidly and the crystalline precipitate will subsequently form. The crystalline precipitate will form early, in case much "B" has been added, later, if less. There is a point, however, where a solution can be made that will give a clear solution in which the crystalline precipitate will not develop for at least 48 hours, and there is a considerable range on either side of this point where solutions quite satisfactory for ordinary use can be made, in which the precipitate will not develop for more than 12 hours under the conditions of the analysis. As a rule, from ¼ to 1 part of "B" to 1 part of "D" is satisfactory. Mix the "B" 20 per cent solution in definite proportions, as follows:

5 cc. "B" + 20 cc. "D" = 1:4.
5 " "B" + 15 " "D" = 1:3.
5 " "B" + 10 " "D" = 1:2.
5 " "B" + 5 " "D" = 1:1.

In each of four small Erlenmeyer flasks put

¼ cc. Benedict's standard, measured roughly.
3 " 5 per cent sodium cyanide.
5 " distilled water.
2 " 10 per cent NaCl in 0.1 N HCl.

To each flask add 1 cc. of the above mixture of "B" and "D". A flocculent precipitate should develop in each flask. Let the flasks stand and watch carefully for the development of a crystalline precipitate. Choose for the final solution that proportion of "B" and "D" which remains *clear* for ½ hour or *more*. If all of them precipitate inside that time, the "D" salt has been improperly prepared. If the solutions are not clear of the flocculent precipitate a still *greater* proportion of "B" should be used. Should all the test flasks precipitate, the dialyzed reagent may, of course, be used without any addition of "B". 5 cc. of this "special reagent" are ample to produce the full color with 1 mg. of uric acid under the conditions of the determination.

Use of Reagents for Determination of Uric Acid in Blood.

The essential points of difference between our modification and the original method are as follows:

1. No sodium carbonate is used, sodium cyanide furnishing the requisite alkalinity.

2. The solution in which the color is to be developed must be diluted to a definite volume.

3. The solutions must be diluted at accurate intervals after the reagent is added.

4. A specially prepared uric acid reagent must be used. A protein-free filtrate is obtained with tungstic acid according to the method proposed by Folin and Wu (6).

For normal bloods, 20 cc. of filtrate, equivalent to 2 cc. of blood, are used, and for bloods suspected of having over 10 mg. of uric acid per 100 cc., 10 cc. or even 5 cc. may be used, if economy of blood be desirable.

To the protein-free filtrate in a 35 cc. centrifuge tube, add 5 cc. of 5 per cent silver lactate in 5 per cent lactic acid. Stir thoroughly with a fine glass rod. Wash the rod into the tube with a few cc. of water, and centrifuge 2 to 3 minutes. The supernatant liquid should be clear. Add a few drops of silver lactate. If a precipitate forms, add 2 cc. more of silver lactate, stir and centrifuge again. Decant the supernatant liquid and drain as completely as possible. Add 2 cc. of 10 per cent NaCl in 0.1 N HCl to the precipitate. Let the solution run into the middle of the precipitate, and not down the side of the tube, a procedure which tends to make the precipitate creep. Break up the precipitate very thoroughly with a fine glass rod.

So far the procedure is that of Folin and Wu. Now add 4.5 cc. of water as accurately as possible with a Folin pipette, and stir again very thoroughly. Wash the rod with 0.5 cc. of water from the Folin pipette. Centrifuge rapidly for 5 minutes. Now transfer the supernatant liquid quantitatively to a 25 cc. flask. This is best accomplished by holding the flask and a fine glass rod, whose tip just touches the side of the flask neck, in the left hand and pouring with the right. When the bulk of the liquid has drained out, touch and retouch the lip of the tube to the rod, until no liquid adheres to the rod as the tube is taken away on several trials. With the rod still in position, run in (down the rod to wash it) 3 cc. of 5 per cent NaCN. This should be accurate to 0.1 cc. Drain the rod and take it out.

Now prepare two standards in 50 cc. flasks. To one flask, add 0.5 cc. of Benedict's standard,[3] to another 1.0 cc. Now add 9.5 and 9.0 cc. of water, respectively, and to each flask 4 cc. of 10 per cent NaCl in 0.1 N HCl, and exactly 6.0 cc. of 5 per cent NaCN.

To the unknown, add 1.5 cc., and to the standards 3 cc. of the special reagent described below. Rotate each flask briskly to insure complete mixture. The additions of the reagent should be made as nearly simultaneously as possible. It is perhaps better to add the reagent at minute intervals and dilute at corresponding times. Let the flasks stand 10 minutes by the clock and dilute to the mark in the same order as the reagent was added. The colorimetric estimations may be made immediately after dilution.

The calculation is made according to the formula:

$$\frac{R \times a \times b}{R_1 \times 2},$$

where R is the reading of the standard, a the figure by which it is necessary to multiply the sample taken to make 100 cc. of blood (50 in case 20 cc. of filtrate are taken), b the amount of uric acid in the standard, expressed in milligrams, and R_1 the reading of the unknown. The equation must be divided by 2, since the unknown is in a 25 cc. flask, while the standard is in a 50 cc. flask.

The color develops slowly, the maximum not being reached in 10 minutes, but the color is sharply proportional to the amount of uric acid present. The depth of color is dependent on the *concentration* of the cyanide in the final solution. Cyanide alone in this concentration gives no color until 8 to 12 hours later, when a

[3] Benedict's standard uric acid solution is prepared as follows: 9 gm. of pure crystallized disodium hydrogen phosphate, together with 1 gm. of crystallized sodium dihydrogen phosphate, are dissolved in 200 cc. of hot water and the solution is filtered, if not perfectly clear. The filtrate is made up to a total volume of about 500 cc. with hot water, and this hot solution is poured upon exactly 200 mg. of pure uric acid, suspended in a few cc. of water in a liter volumetric flask. The mixture is agitated for a moment or two, until the uric acid completely dissolves, and then cooled. Exactly 1.4 cc. of glacial acetic acid are added, and the flask is diluted to the mark and mixed. 5 cc. of chloroform are then added to prevent the growth of bacteria or molds. 5 cc. of this solution contain exactly 1 mg. of uric acid. This solution keeps perfectly well for at least a month.

faint tinge of blue develops. A much greater concentration of cyanide will develop a blue color without uric acid. The color continues to increase very gradually over a period of many hours. The increase is proportional in the standard and the unknown. No carbonate is used. Folin's uric acid standard cannot be used since the presence of sulfite prevents the development of the deep color If *serum*, rather than whole blood, be used, the determination may be made after the plan for urine (see below). We have been unable thus far to find polyphenols in *serum*. That portion of the precipitate from silver lactate which remains behind after liberation of the uric acid with HCl has never given any color with the reagent when serum was used. Apparently, the polyphenols are confined to the cells.

Use of Reagents for the Determination of Uric Acid in Urine.

The same principles of modification apply to the analysis of urine as to the analysis of blood. Very careful control of dilution and accurate time intervals are required and again cyanide is the only alkali used.

Add to 1 or 2 cc. of urine according to concentration in a 35 cc. centrifuge tube 6 cc. of water. Then add 5 cc. of silver lactate, stir and centrifuge. Decant the supernatant liquid. To the precipitate add exactly 4 cc. of 5 per cent NaCN and exactly 10 cc. of water. Stir until all the precipitate is dissolved.

Prepare in two 100 cc. flasks suitable standards. To one flask add 2 cc. of standard, to another 4 cc. of standard and 8 and 6 cc. of water, respectively. To each standard add 4 cc. of cyanide. Then with time intervals as with blood, add 5 cc. of special reagent to each flask, and to the unknown in the tube. Mix thoroughly. Allow each to stand exactly 10 minutes, and dilute to the mark. The unknown can be poured into the 100 cc. flask just before the 10 minutes are up and the washings serve to dilute it at the right time.

Using 2 cc. of urine in a 100 cc. flask, and 2 and 4 cc. of standard in a 100 cc. flask, a range of from 0 to 60 mg. of uric acid per 100 cc. is covered. With 1 cc. of urine and the same standards a range of from 60 to 120 mg. per 100 cc. is covered.

If there is albumin in the urine, it must be removed by heat and acetic acid, or tungstic acid, as even very small amounts of albumin prevent the proper precipitation of uric acid by silver lactate.

EXPERIMENTAL.

When phosphotungstate "B" is used alone as the reagent for uric acid a crystalline precipitate develops very quickly; a flocculent precipitate develops when phosphotungstate "D" is used alone. On the other hand, the effect on the formation of a precipitate of combination of phosphotungstate "B" and "D" is quite striking for it has been possible to find a proportion between the two salts which keeps the solution clear (Table I). The proportion of the two salts which gives the best results may vary with the different lots.

The depth of color is dependent upon the concentration of the cyanide radical. The tendency to precipitate is determined by

TABLE I.

Effect of Varying Amounts of "B" and "D' on Time of Appearance of Precipitate.

Proportion "B" to "D."	Precipitate appeared.
1:0	3½ minutes.
4:1	16 "
2:1	22 "
4:3	28 "
1:1	60 "
1:3	No precipitate.
0:1	Always cloudy. No crystalline precipitate.

the OH ion concentration. From these facts it might be inferred that Folin's original solution could be used with NaCN as the only alkali. But while increased color and freedom from precipitate can be obtained in this way, such a large experimental error is introduced that the method is quite worthless, except when the readings of the standard and the unknown are very close together. This is shown in Table II. Method 1 indicates the method described in this paper. Method 2 indicates the use of Folin's reagent with NaCN as the only alkali. Method 3 indicates the regular method of Folin and Wu.

In the above experiments, the same blood filtrate was used throughout and varying amounts were taken for analysis in order to vary the readings of the unknown in comparison with that of the standard. It will be seen that unless the readings are close

together very large errors are introduced when Folin's solution is used with cyanide alone.

TABLE II.

Comparison of Methods Using NaCN as the Only Alkali.

Method.	$\frac{\text{Reading of standard}}{\text{Reading of unknown}}$.	Amount of uric acid.
		mg. per 100 cc.
1	$\frac{20}{22}$	3.0
2	$\frac{20}{22}$	3.0
3	$\frac{20}{22}$	3.0
1	$\frac{20}{33.5}$	2.9
2	$\frac{20}{44.0}$	2.2
1	$\frac{20}{13.4}$	3.0
2	$\frac{20}{9.5}$	4.2

TABLE III.

Rate at Which the Color Develops.

Solution made.	Diluted.	Interval.	Reading.
a.m.	*a.m.*	*min.*	
10.45	10.50	5	20.0
10.47	10.53	6	20.0
10.49	10.56	7	20.0
10.50	11.00	10	20.0
11.45	11.50	5	20.2

Development of the Color.

The color develops slowly and flasks should stand before dilution for 10 minutes. The rate of increase of color has by this

time fallen off so much that a considerable error in time elapsed before dilution does not introduce an appreciable variation in the reading, as may be seen in Table III.

Standards and unknowns made up in 1 hour are comparable with one another.

Solution made.	Reading.
12.15	20.0
12.45	20.0
1.00	19.9
2.15	21.6

The intensity of the color is, as we have said, dependent on the concentration of the cyanide radical. A considerable variation in cyanide concentration, however, is consistent with accurate readings. Flasks containing 1 cc. of standard, 5 cc. of water, and 1.5 cc. of reagent were made alkaline with varying amounts of cyanide. See Table IV.

TABLE IV.

Effect of Varying Amounts of Cyanide.

Cyanide.	Reading.
cc.	
3.0	20.0
3.1	19.9
3.2	20.0
3.5	20.0
4.5	18.9

From the foregoing table, it is evident that when the method calls for 3 cc. of NaCN, a variation of ± 0.5 cc. does not alter the result. However, in our work we measure the cyanide solution to within ± 0.1 cc.

Sodium carbonate or other strong alkali prevents the development of the deep color, and sodium sulfite lessens the color even more, so that Folin's sulfite standard cannot be used in our method. The color develops slowly and does not reach a maximum for an hour or more, but there is a sharp proportionality between the amount of uric acid and the depth of color.

Accuracy.

We have taken as our standard of accuracy, Folin's method. The method we propose, we believe is quite as accurate as Folin's. The analyses in Table V, taken from a large series of determinations, serve as examples.

TABLE V.

Folin's Method Compared with That of the Authors, Milligrams of Uric Acid in 100 Cc. of Blood.

Folin's method.	Authors' method.
23.3	23.2
15.0	15.0
15.2	15.0
3.0	3.0
3.4	3.3

Specificity.

The reagent, prepared as we have suggested, reacts towards organic reducing substances in alkaline solution exactly as does Folin's solution.

Composition of Phosphotungstates.

We have been unable to make satisfactory analyses of the two salts used in preparing the reagent. Our attempts in this direction have convinced us that neither is a pure chemical substance. Both give indications of being mixtures of two or more compounds.

Sodium phosphotungstate "B" can be easily recrystallized from water or alcohol. Sodium phosphotungstate "D" is very difficult to purify and seems to crystallize fractionally as if it were composed of several compounds. We have prepared the free acid from sodium phosphotungstate "B". Furthermore, we have prepared in a comparatively pure state, the blue reduced tungstate. It seems to be stable in air and is blue-black in color. This was prepared by evaporating a solution of the salt in the presence of metallic zinc in an evacuated desiccator containing a strong solution of pyrogallic acid in strong NaOH.

SUMMARY.

A modification of Folin's method for the determination of uric acid in blood and urine is described. In our hands, this modification eliminates the two disadvantages, the faint color, and the precipitate in the final solution for colorimetric estimation, of the original procedure.

The color developed by our method is nearly five times as great as that developed on Folin's method, and does not fade over a period of several hours. In fact the color increases gradually and proportionally in both standard and unknown, so that when standard and unknown are made up at the same time, they may be read at any time during the next 2 hours or more. No crystalline precipitate develops in the colored solution.

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AMINO-ACIDS IN NUTRITION.

IV. A MODIFIED BIOLOGICAL METHOD OF STUDYING AMINO-ACID DEFICIENCIES IN PROTEINS. CYSTINE AS A GROWTH-LIMITING FACTOR IN THE PROTEINS OF THE GEORGIA VELVET BEAN (STIZOLOBIUM DEERINGIANUM).

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(Received for publication, October 19, 1921.)

Employing essentially the technique of McCollum and Davis (1), using the seed as the only source of protein, and satisfying all the other dietary essentials in the rations, the author attempted to make amino-acid additions to the pea (*Vicia sativa*) as a part of his general scheme of studying the capacity of the animal organism to synthesize the pyrrolidine nucleus of the protein molecule (2), but in that study has met with no positive results. This paper will show, by a modified method of procedure, the practicability of making amino-acid additions to such complex substances as seeds.

Waterman and Jones (3), in a recent communication, state that amino-acid deficiencies in the Chinese and Georgia velvet beans cannot account for the failure to promote growth; for analyses (4, 5) have shown the above mentioned proteins to be adequate except possibly with respect to cystine; and no improvement resulted from the addition of this amino-acid.

It will be shown in the following pages by the modified procedure adopted by the author that *cystine is unquestionably a growth-limiting factor in the proteins of the Georgia velvet bean*, which could not have been found by employing previous methods of making straight amino-acid additions. The results of the experiments are given in the following charts.

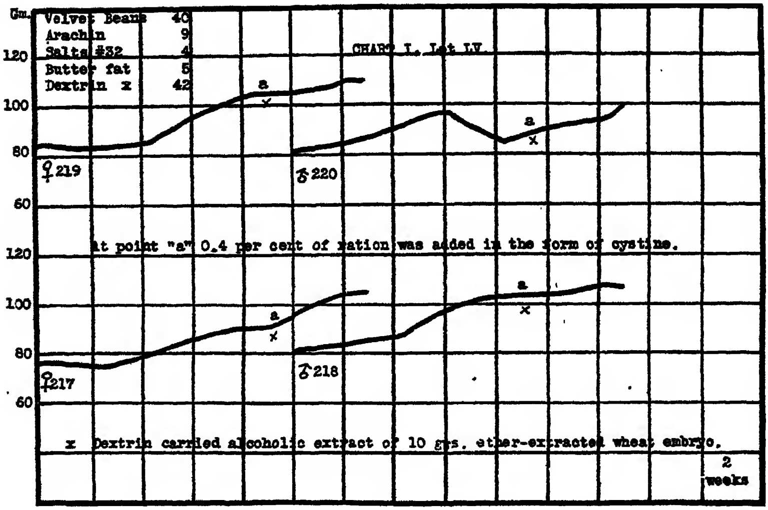


Chart I, Lot LV. This chart indicates that when Georgia velvet beans are fed as the only source of protein at a level of 40 per cent, very little growth takes place, and that arachin, one of the globulins and the main protein from the peanut, does not furnish amino-acids to supplement those deficient in the velvet beans. At point "a" 0.4 per cent of the ration was added in the form of cystine, but no response was obtained.

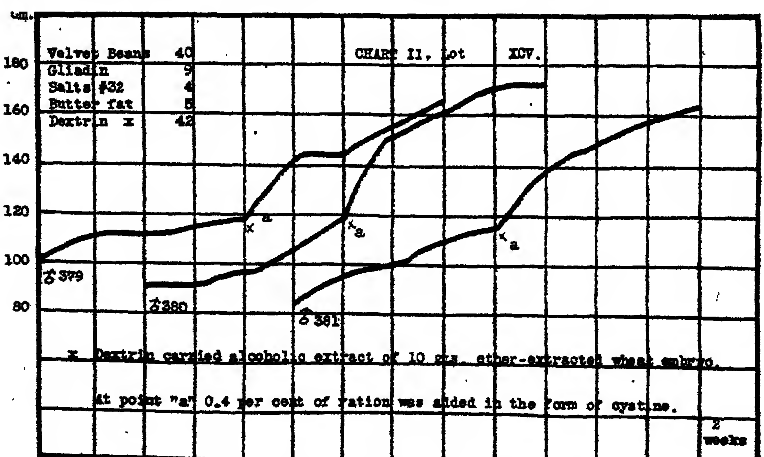
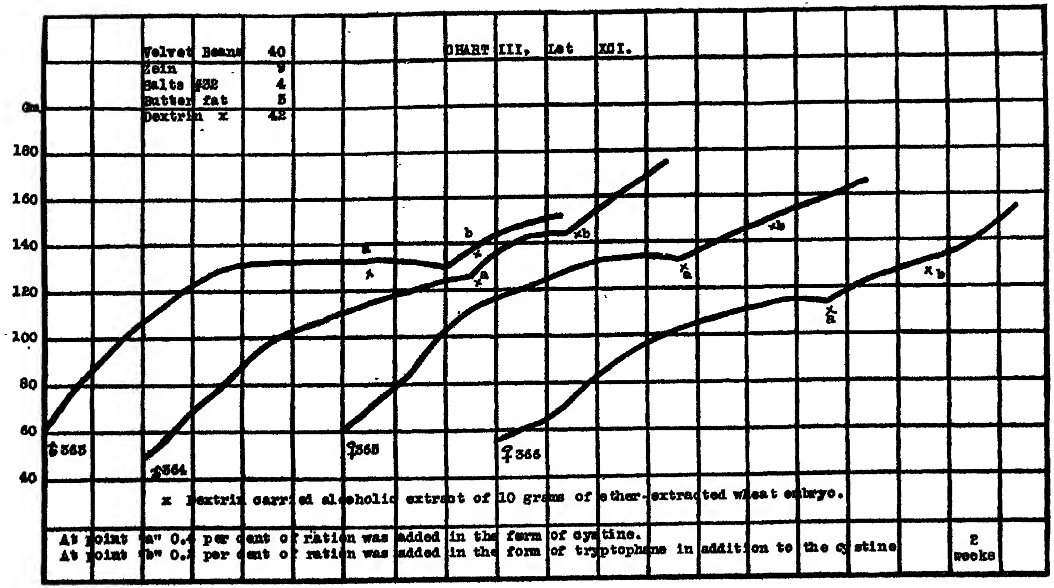


Chart II, Lot XCV. This experiment demonstrates that, in the presence of gliadin, there is a definite response to cystine addition to the velvet bean proteins, which is not, however, very marked 2 weeks after this amino-acid addition.



'CHART III, LOT XCI. Zein seems to supplement the proteins of the Georgia velvet bean in the earlier periods of growth, but, it will be noted that after 8 weeks the nature of growth is considerably retarded. The addition of cystine at point "a" brought about a slow but definite improvement in growth with no further increase in the character of growth on the addition of tryptophane to the cystine at point "b". This experiment and the preceding, Lot XCV, strongly suggest that cystine is a growth-limiting factor in the proteins of the Georgia velvet bean, which becomes apparent only after other amino-acids are satisfied as supplied by such deficient proteins as gliadin and zein.

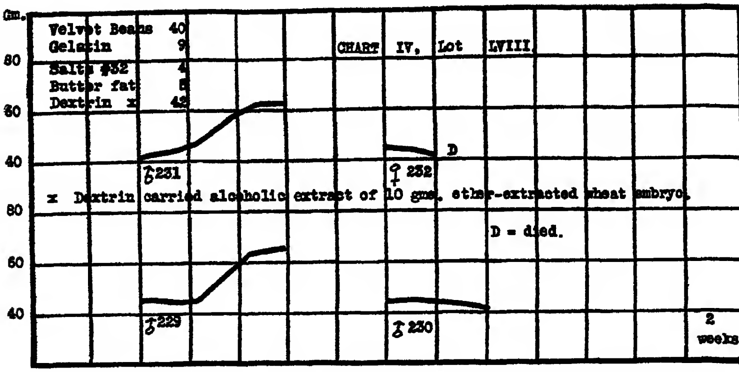


CHART IV, LOT LVIII. The behavior of the animals on this ration certainly indicates that gelatin does not furnish the necessary missing links deficient in the proteins of the velvet bean.

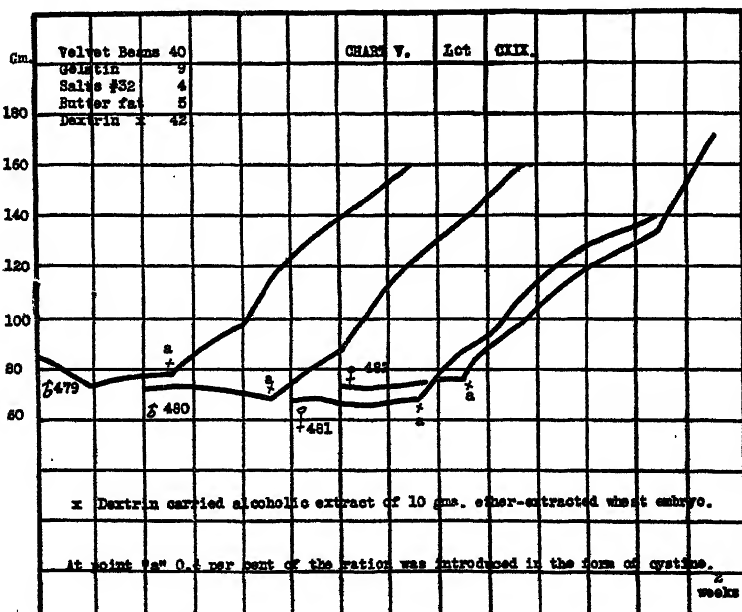


CHART V, LOT CXIX. This is a duplicate of the preceding experiment, Lot LVIII. The experiment was repeated using animals a little heavier, so that they could better maintain themselves on the poor velvet bean-gelatin ration, and in order to have an opportunity to make a cystine addition. After the adding of 0.4 per cent of the ration in the form of cystine,

the writer found, to his great surprise, that all the animals, although they have previously failed to make any growth (Chart IV, Lot LVIII) and produced only maintenance curves on the same ration in this experiment, have begun to grow in a very marked manner, and have made excellent growth for a period of 10 weeks after the amino-acid addition, after which time the experiment was discontinued. This experiment, then, furnishes conclusive evidence that, providing other amino-acids in the form of the deficient protein (gelatin) are supplied, cystine shows itself up remarkably as one of the determining growth-limiting factors in the proteins of the Georgia velvet bean.

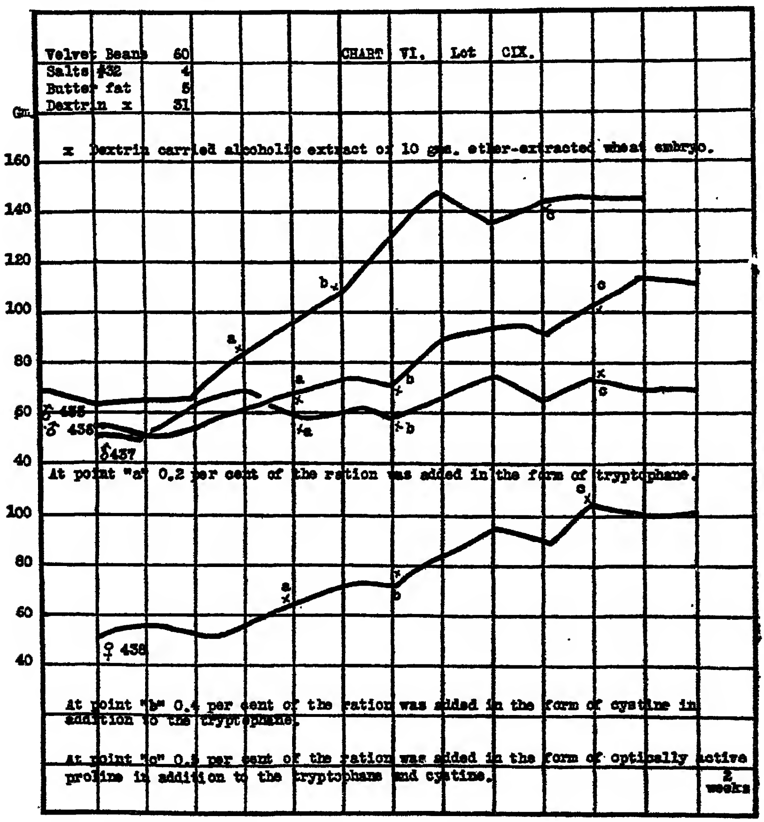


Chart VI, Lot CIX. This ration illustrates that even when the Georgia velvet bean proteins are fed at as high a level as 60 per cent, which would furnish 16.5 per cent protein, very little growth takes place. At point "a" 0.2 per cent of the ration was added in the form of tryptophane, but without any response. At point "b", after the addition of cystine to the extent of

0.4 per cent of the ration, Animals 436 and 438 showed only a slight improvement in growth. Rat 435 shows little change in the slope of the curve, and Rat 437 shows no response at all. It is certainly not convincing from this experiment that cystine is a growth-limiting factor in the proteins in question. The introduction of proline in the ration at point "c" in addition to the tryptophane and cystine brought no response.

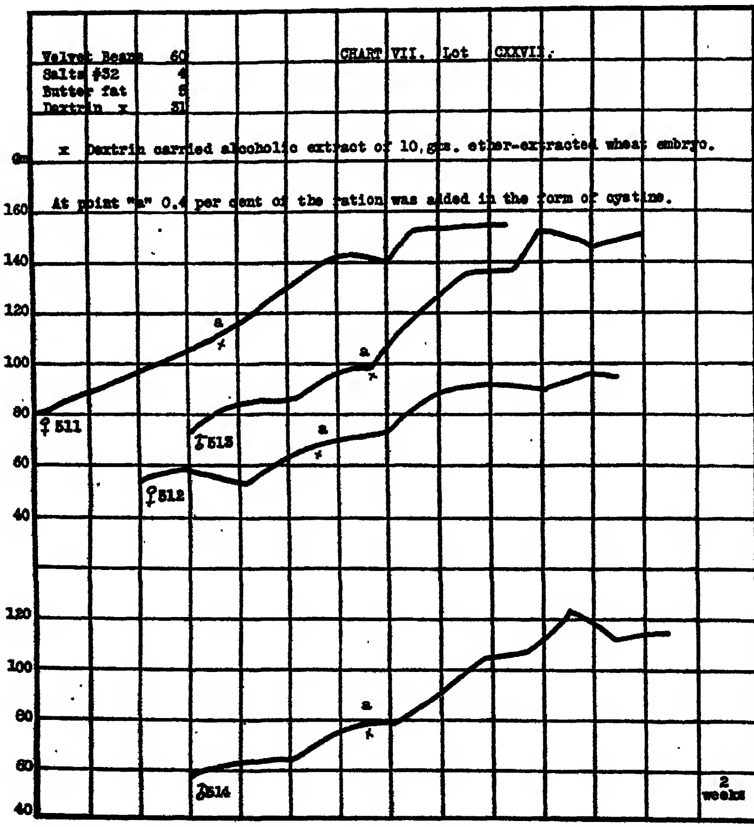


Chart VII, Lot CXXVII. This experiment is a duplicate of the one just preceding, Lot CIX, the purpose being to make the addition of cystine in the absence of tryptophane. There seems to be some response to cystine in the case of Animals 513 and 514, but certainly very little, if any, in the case of Rats 511 and 512. On the whole, judging from the character of amino-acid responses of this lot and the preceding lot, No. CIX, it seems that the suggestive response to cystine takes place to the same extent in the absence of tryptophane.

DISCUSSION.

Previous work (6) on the biological analysis of the seed of the Georgia velvet bean indicated that 40 per cent is the optimum plane of seed intake; therefore, 40 per cent was the level chosen for the study of the quality of proteins of this bean from the standpoint of amino-acid deficiencies. Chart I, Lot LV indicates that the addition of cystine to the proteins in question fed at the optimum level, even in the presence of arachin, brings about no response, and one might conclude from this experiment that cystine is not a growth-limiting factor in the proteins of the Georgia velvet bean. Further work, however, showed that when cystine is added to a 40 per cent level of the seed in the presence of gliadin or zein, definite, although not very marked, improvement in the character of growth takes place. The addition of cystine, however, to a velvet bean-gelatin ration, on which diet only maintenance takes place, is immediately followed by considerable growth which is continuous. The addition of cystine in the presence of the above mentioned deficient proteins was made with the following idea in mind. Picturing the possibility that the proteins of a seed may owe their deficiency to a number of amino-acids as represented by a, b, c, d, e, f, and g, and supposing we are interested to know if *g* representing *cystine*, is a growth-limiting factor; also supposing that a, b, and c, belong to the monoamino group which the animal organism may be able to synthesize readily, then amino-acids d, e, and f will still have to be supplied before a response to *g* may be obtained. This furnishes the basis for the addition of the deficient proteins between the seed and the amino-acid. Evidently gliadin and zein are furnishing some amino-acids in which the proteins of the Georgia velvet bean are lacking but not to the extent that gelatin is; hence, the more marked response in the presence of gelatin.

In a former communication (2) the author stated that Mr. Koehler of the Laboratory of Agricultural Chemistry, University of Wisconsin, very carefully analyzed zein, purified by reextracting three times with 70 per cent alcohol, and could find no cystine in it. On improving his technique for the study of the cystine content of proteins after hydrolysis, Mr. Koehler has found

(7) that a great portion of the organic sulfur of zein is in the form of cystine, also that casein is much higher in cystine than formerly reported. In view of this work, it is possible to see why fairly good growth was obtained during the first 8 weeks on a zein-velvet bean ration, and that later a response to cystine addition was secured.

Although previous work indicated that 40 per cent is the optimum level of velvet bean seed intake, an attempt was made to add cystine to a 60 per cent plane of intake, in order to see if the amino-acids from 20 per cent additional seed could replace 9 per cent of any of the deficient proteins employed in this investigation. It will be noted, however, that while some response is suggestive in the case of certain animals of the duplicate lots, Nos. CIX and CXXVII, the results are not at all conclusive; and the data clearly indicate that without the use of the deficient proteins it would have been impossible to find that cystine is a determining growth-limiting factor in the proteins of the Georgia velvet bean, since Waterman and Jones (3) have failed, just as the writer has in his preliminary work, to secure a response to straight cystine additions to the velvet bean seed.

The addition of tryptophane and proline in the presence of cystine brought about no response.

A problem that suggests itself in connection with this work is: What amino-acids is gelatin furnishing to the proteins of the velvet bean that are essential before a response to cystine can be secured. This study is under investigation and will be reported later.

Preliminary work on amino-acid deficiencies in edestin (the globulin from hemp seed (8)) indicated that certain amino-acids are deficient in that protein, but the work was not at all conclusive. Employing the improved technique described in this paper to explore further the cause of the poor nutritive quality of edestin, the author has obtained corroborative evidence of the earlier suggestive data. The results of the experiments on amino-acid deficiencies in edestin will appear in a following communication.

SUMMARY.

1. The proteins of the Georgia velvet bean are deficient in character.

2. Arachin does not supplement the proteins in question; neither is there a response obtained to the addition of cystine in the presence of arachin.

3. There was no response to the addition of proline even in the presence of cystine and tryptophane.

4. A slow but definite response to cystine was secured in the gliadin-velvet bean and the zein-velvet bean rations.

5. A very marked and continuous response to cystine was obtained in the gelatin-velvet bean maintenance ration.

6. *Cystine is a determining growth-limiting factor in the proteins of the Georgia velvet bean, a fact which becomes apparent only in the presence of such deficient proteins as gliadin or zein, and most markedly in the presence of gelatin.*

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AN ORGANIC CONSTITUENT OF THE TUBE OF MESOCHÆTOPTERUS TAYLORI, POTTS.

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(Received for publication, October 20, 1921.)

Mesochætopterus taylori is a polychæte worm which is known to occur only on the northwest coast of North America. It has been described in detail by Potts (1). It may be found in the neighborhood of Nanaimo, wherever a stretch of clean sand is exposed at low tide, inhabiting a long tube constructed of thin but tough material in which sand is imbedded.

The writer (2) has recently made pentose determinations in a number of substances of marine animal origin, employing Grund's modification of the method of Tollens and Kröbe (3). Of all those examined the material of which the tube of *Mesochætopterus taylori* is composed gave the highest percentage yield of furfural and the presence of a large quantity of a pentose derivative was suspected. All the pentose compounds which had previously been encountered in animal material were constitutents of organized tissues, were soluble in water, and consisted either of nucleotides or β-nucleoproteins. It seemed *a priori* improbable that a compound of either of these classes should occur in a secreted material like the tube of a worm and their absence was confirmed when the furfural-yielding constituent was found quite insoluble in water.

The nature of this constituent seemed worth investigating. It was subsequently found that more than one furfural-yielding substance was involved. The present communication deals with one of them. It has not been fully characterized, since neither sufficient material nor adequate facilities were available, but there seems little doubt as to the class of compounds to which it belongs.

EXPERIMENTAL.

The animals were carefully removed from the tubes and the tubes, after washing as free from sand and debris as possible, dried first at air temperature and finally at 100°C. More sand was removed from the dry substance by grinding and sieving, but a certain amount remained imbedded in the substance of the tube.

The material thus prepared contained 32.3 per cent of ash and yielded 3.5 per cent of furfural on treatment with 12 per cent hydrochloric acid. 140 gm. (corresponding to 400 to 500 tubes) were covered with a 2 per cent solution of sodium carbonate and kept between 40 and 50°C. for 24 hours. On making the alkaline extract distinctly acid with hydrochloric acid a close grained flocculent precipitate was produced. An excess of alcohol was added and the precipitate filtered off, washed with alcohol, and dried at 100°C. It yielded furfural on boiling with 12 per cent hydrochloric acid, gave slight xanthoproteic and biuret reactions, and was evidently not of the same character as the precipitate obtained from subsequent extractions. It was accordingly held separately from these. Subsequent extractions yielded on acidification a heavy bulky transparent gelatinous precipitate. After addition of alcohol this was easily filtered off through muslin, but, owing to its colloidal nature, could not be washed entirely free from hydrochloric acid. The tube material was extracted five times for 24 hour periods and the precipitates were accumulated in alcohol. The fifth extraction did not yield very much precipitate and the process was discontinued. The residual substance, after washing free from sodium carbonate, and drying, weighed 77 gm.; it contained 24.4 per cent of ash and yielded 2.5 per cent of furfural, so that, either the extraction was less complete than seemed to be the case from the small quantity of material obtained by a repetition of the treatment, or more than one furfural-yielding body was involved. The latter proved to be the case since it was found that such a body could be extracted by 1 per cent sodium hydroxide, after which the residue yielded only a trace of furfural. The substance obtained by neutralization of the sodium hydroxide extract was of an entirely different physical character to that which forms the subject of this paper and its further examination has yet to be made.

The substance precipitated by acidification of the sodium carbonate extract bore a very close superficial resemblance to the "alginic acid" which has been isolated from a number of marine algæ and was at first taken for it or a similar pentosan derivative. It was extractable in the same way and altered in physical properties if extraction were carried out at a higher temperature than 50°C. Like alginic acid it consisted of a colloidal substance capable of absorbing several times its own weight of water, readily soluble in dilute alkali when moist, but becoming hard, horny, and resistant to solvents when dry, forming insoluble salts with the heavy metals, soluble ones with the alkalies, and yielding furfural in considerable quantity on treating with 12 per cent hydrochloric acid. It gave no protein reactions, but contained organic nitrogen.

The method employed for the purification of alginic acid by Hoagland and Lieb (4) was tried on the substance. The crude material was well washed with alcohol, freed from alcohol as far as possible by suction, dissolved in the least possible amount of warm 2 per cent sodium carbonate, and poured into alcohol. This precipitated the sodium compound in the form of non-gelatinous strings drying to a brownish tough horny mass swelling enormously and eventually dissolving in water, precisely like the corresponding salt of alginic acid. 5 gm. of the sodium salt were dissolved in water and dialyzed in a collodion bag in running water for a week. At the end of this time a sample, removed and acidified, yielded material still containing 17.7 per cent of ash and nitrogen. The dialysis was therefore continued for another week, but no appreciable reduction of ash constituents resulted and the nitrogen reaction persisted. The main solution was therefore acidified which produced a solid jelly. This was broken up as far as possible in water and the suspension returned to the dialyzer. After 10 days most of the hydrochloric acid had been removed and only a slightly viscous solution remained which yielded very little precipitate on adding alcohol either before or after acidification. The free acid substance had either decomposed or diffused through the membrane on removal of the bulk of the hydrochloric acid. In the light of knowledge of the composition of the substance subsequently obtained the former is the more likely alternative. In respect of this inability

to purify by dialysis the substance differed essentially from alginic acid.

The remainder of the sodium salt was dissolved in water and the free acid reprecipitated with hydrochloric acid. This was dissolved in 2 per cent sodium carbonate and the sodium salt again precipitated by alcohol. After washing with alcohol and drying at 100°C. it weighed 40 gm., contained 33.04 per cent of ash, and yielded 4.1 per cent of furfural.

Acid Hydrolysis.

The sodium salt yielded a solution with strong reducing action on treating with mineral acids. It was, however, very resistant to acid hydrolysis; 50 hours heating in a boiling water bath with 6 per cent hydrochloric acid was necessary to attain a maximum reducing power. Weaker acid was almost without action. Stronger acid led to excessive carbonization accompanied by production of furfural, and heating beyond 50 hours led to decrease of reducing action.

10 gm. of sodium salt were dissolved in 50 cc. of water and 50 cc. of 12 per cent hydrochloric acid added. After heating in a boiling water bath for 50 hours the solution had a reducing action indicating a yield of 24.4 per cent of reducing sugar (calculated as dextrose). This was far in excess of the reducing action to be anticipated from a pentose calculated from the furfural yield and it was clear that, either the furfural was not produced from a pentose derivative, or some other reducing substance, in addition to the furfural-yielding one, was produced by acid hydrolysis. That the latter alternative was true was shown by the fact that on distilling the substance with 12 per cent hydrochloric acid until no further furfural came over, the residue still had a powerful reducing action. It seemed also true that the production of furfural was not due to a pentose derivative, for, on testing the solution of the fully hydrolyzed substance for free pentose with Bial's orcinol reagent there was no reaction and, on making the acid strength of the solution up to 12 per cent and distilling, no furfural was obtained. The furfural-yielding substance had therefore been destroyed during the hydrolysis.

The only substances known to occur naturally and to yield furfural in considerable quantity on treating with 12 per cent

hydrochloric acid, in addition to pentose compounds, are the oxycelluloses, galacturonic and glucuronic acids, and their derivatives. The first two classes of compounds have hitherto been identified with certainty only in vegetable material. Glucuronic acid seemed therefore to be the most probable furfural-yielding compound to be involved in the substance under discussion. The origin of the substance added to the probability since the worm-tube is undoubtedly constructed from a secretion of a mucoid nature. Chondroitin sulfuric acid and allied compounds have been prepared from various mucoids and, in at least one case, that of chondromucoid, it has been shown by Levene and La Forge (5) that the chondroitin sulfuric acid contains glucuronic acid as one of its components. Its physical properties, its resistance to acid hydrolysis, and the simultaneous destruction of the furfural-yielding residue under the treatment, also point to a compound of the nature of chondroitin as the source of the furfural. Since the component believed to be glucuronic acid was broken down by ordinary methods of hydrolysis and sufficient material was not available to attempt the method used by Levene and La Forge to isolate it from chondroitin, glucuronic acid has not been positively identified. Its presence is inferred from the foregoing argument and the identification of other constituents similar to those of chondroitin.

Preparation of Phenylosazone.

The remainder of the solution resulting from the acid hydrolysis was boiled with a little animal charcoal and filtered. The filtrate was neutralized with sodium hydroxide, evaporated to small volume, and poured into about 5 volumes of alcohol which threw down a resinous substance. The solution was separated from this and evaporated, the residue taken up with alcohol, again evaporated, and finally taken up with about 25 cc. of water. 1.5 gm. of phenylhydrazine hydrochloride, 2.5 gm. of sodium acetate, and 2.5 cc. of glacial acetic acid were added and the whole was heated in a boiling water bath. After heating for 2 hours only a small quantity of black resinous material had separated. On filtering this off and cooling the filtrate a small quantity of a fine crystalline precipitate formed. This was filtered off and the mother liquor heated for another hour, which led to fur-

ther precipitation. Finally, on concentrating the liquor somewhat, a third crop of crystals was obtained. The first fraction was recrystallized from hot water and, after drying, melted at 173–174°C. After a second recrystallization with a little alcohol present it melted at 178–179°C. The second fraction, after recrystallization, melted fairly sharply at 179°C. and the third at 182–184°C. It seemed, therefore, that all three fractions consisted in the main of the same compound and that its melting point was somewhere between 179 and 184°C. This is very near the melting point (180–185°C.) of the osazone originally obtained by Levene and La Forge (6) from chondrosamine derived from chondroitin, though in a later paper Levene (7) shows that, if sufficiently purified, their osazone melts at 201°C. and is identical with galactosazone.

Levulinic Acid.

10 gm. of material were heated in a boiling water bath with 100 cc. of 18 per cent hydrochloric acid for 22 hours under a reflux condenser. After boiling to insure freedom from furfural the solution was filtered and extracted four times with ether. The ethereal extracts were combined and evaporated leaving a small brown syrupy residue which was heated gently until free from volatile acid and dissolved in a little water. The solution gave a good iodoform reaction on adding a drop to a warm alkaline solution of iodine. Zinc oxide and a little animal charcoal were added and the solution was boiled and filtered. On standing a small quantity of crystalline salt separated. This was filtered off, washed with alcohol and ether, and dried. Slight charring took place on drying. The dry salt dissolved only partially on boiling with water, leaving a brown residue. Addition of silver nitrate to the solution produced a small precipitate which dissolved on boiling, with formation of a little silver oxide. The filtrate from this on standing in the dark deposited a crystalline precipitate, but the amount was too small to characterize further.

The presence of levulinic acid in the solution, and thus of a hexose or hexose derivative in the original substance, is indicated by the above tests. Levene and La Forge (8) point out that a good yield of levulinic acid can be obtained from chondrosin only after previous deamination. Omission of this preliminary treatment probably accounts for the small amount obtained.

Attempt to Isolate Hexosamine.

The presence of an amino compound was indicated by the fact that the solution of the substance evolved ammonia very readily on warming with sodium hydroxide and contained no protein or ammonium salt. It was confirmed by obtaining nitrogen on treating the solution with acetic acid and sodium nitrite. An attempt was made to isolate the amino compound as hydrochloride. 5 gm. of the sodium salt were ground as finely as possible and boiled with 25 cc. of concentrated hydrochloric acid until furfural evolution ceased and nothing but a small quantity of charred material remained undissolved. This was filtered off, and the filtrate decolorized with animal charcoal and evaporated to small volume. Nothing separated on standing. On pouring into a large volume of alcohol a white amorphous precipitate separated. This was filtered off, washed carefully with alcohol and ether, and dried in a desiccator. It dried to resinous lumps readily soluble in water. The solution reduced strongly evolved ammonia on treating with sodium hydroxide and gave a phenylosazone, but a crystalline hydrochloride could not be separated from it. Levene and La Forge (8) mention the difficulty of obtaining the hexosamine hydrochloride from chondroitin sulfuric acid using the crude sodium salt and were successful only when employing a purified barium salt. Unfortunately the relation of the substance under investigation to chondroitin sulfuric acid did not become apparent until most of the raw material was used up and the height of the tides made it impossible to obtain more.

Detection of Sulfuric and Acetic Acids.

The aqueous solution of the substance gave a colloidal precipitate of the barium salt of the complex acid on addition of barium chloride. After treating the solution with hydrochloric acid barium sulfate was precipitated on addition of barium chloride. It was clear, therefore, that the compound contained organically combined sulfuric acid.

The detection of acetic acid was less certain. On distilling 5 gm. of material with 50 cc. of 25 per cent sulfuric acid a strongly acid distillate was obtained. The distillation was continued,

keeping the volume constant in the reaction flask meanwhile by additions of water, until the distillate was only slightly acid. The distillate was neutralized with sodium hydroxide and evaporated to dryness. The residue gave doubtful cacodyl and ethyl acetate reactions, but unquestionably consisted of a salt of an organic acid.

CONCLUSION.

The substance extracted from the tube of *Mesochætopterus taylori* by warm 2 per cent sodium carbonate has thus been shown to contain, besides a furfural-yielding substance, probably glucuronic acid, a hexosamine, probably galactosamine, sulfuric acid, and a volatile organic acid. It thus bears a close resemblance to the chondroitin sulfuric acid from chondromucoid investigated by Levene and La Forge. To determine whether it is identical with that compound a great deal more material would be necessary.

Furfural has been obtained in approximately the same proportion as is yielded by the tube of *Mesochætopterus taylori* from the tubes of a local *Spiochætopterus* and *Sabella* and from that of *Chætopterus variopedatus*. It is likely, therefore, that similar compounds exist in the tubes of these worms. A complex of like nature seems to have been described by Kelly (9) from the tube of *Spirographis*. It is therefore probable that the occurrence in worm-tubes is general.

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CURVE OF SUGAR EXCRETION IN SEVERE DIABETES.

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The work reported in this paper represents a study of the total quantities of sugar excreted in the urine of a number of diabetic patients during periods in which they were being held in the non-diabetic status, or what is sometimes called the "sugar-free" condition, by dietary restriction, and during the periods in which the diets were gradually increased until clearly abnormal quantities of sugar appeared in the urine. The purpose has been to plot the curves of glucose excretion by diabetic patients on diets increasing gradually from those on which they show no abnormal glycosuria up to the point at which definitely abnormal glycosuria is induced. The total sugar excreted in the urine every 24 hours was determined quantitatively by the method described by Benedict and Osterberg[1] for the quantitative determination of sugar in normal urine.

The glucose equivalents of the diets were calculated as the utilizable carbohydrate plus the protein $\times$ 0.58 plus the fat $\times$ 0.1 as per the equation $G = C + 0.58P + 0.1F$[2] in which G = glucose, C = carbohydrate, P = protein, and F = fat. The total glucose supply to the organism was not always represented entirely by the diet since in most instances the diets were at times below maintenance values, necessitating the catabolism of protein and fat from the tissues. In some instances also, the quantity of fat in the diet could have exceeded the quantity actually catabolized.

Case 1.—(Miss R.) Age 60 years. Weight 38.6 kilos. On admission to the hospital she showed marked denutrition, glycosuria, acidosis, alkali deficit, and symptoms of acid intoxication. She was given alkali and

[1] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

[2] Woodyatt, R. T., *Arch. Int. Med.*, 1921, xxviii, 125.

reduced to the non-diabetic status by dietary restriction. On a diet of 32 gm. carbohydrate, 57.5 gm. protein, and 53 gm. fat—containing 835 calories and the equivalent of 71 gm. glucose (*G*), the total sugar eliminated each 24 hours for 3 days remained constantly at a little over 400 mg. per day (see Chart 1). The diet was then increased very slowly; and on 35 gm. carbohydrate, 60 gm. protein, and 60.5 gm. fat—924 calories ($G = 76$), the sugar excreted per day for 2 days was no higher than it had been on the 835 calorie diet. On the 3rd day, however, while the patient was still on the same diet, the sugar eliminated suddenly increased to 1,050 mg. and a subsequent addition equivalent to 4 gm. glucose and 34 calories resulted

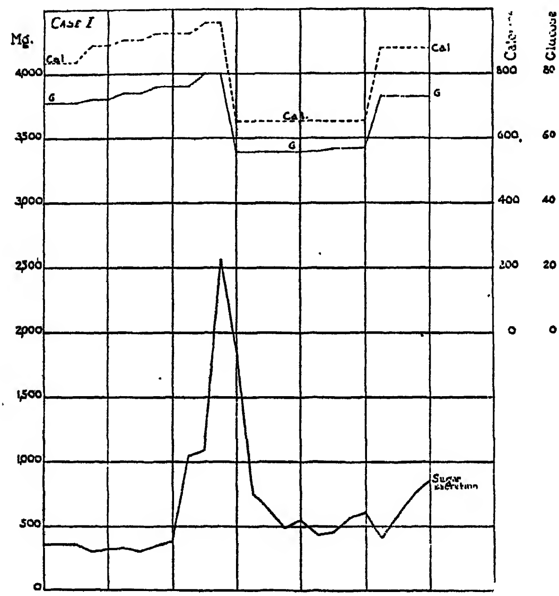

CHART 1.

in the excretion of 1,092 mg. for the first 24 hours and 2,556 mg. for the second 24 hours on which the same diet was maintained. Without entering into a discussion of the factors which may have determined this sudden acceleration of the glucose excretion in this particular case, it affords an example of the suddenness with which the sugar excretion may jump from a normal level to an abnormally high level.

Case 2.—(Mr. M.) A young man, 26 years of age, weighing 50 kilos, who entered the hospital in severe acidosis and denutrition and who had been "desugarized" by a diet consisting of green vegetables and broth aggregating 298 calories ($G = 27$), excreted 520 mg. of sugar on the first day of observation. Thereafter, the sugar excreted in the urine remained

practically constant from day to day during the time in which the diet was increased from a caloric value of 298 to one of 1,336 calories, the G for the diet rising from 27 to 116 gm. Thus, the total quantity of sugar eliminated for the 24 hour period in which the diet contained 398 calories with $G = 51$, was 680 mg. and it was only 683 mg. on a 1,236 calorie diet with $G = 101$ gm. The next addition, which brought the diet up to 1,404 calories, and increased G to 123 gm. (an increase of 22 gm.) resulted in a total urinary sugar excretion of only 1,006 mg.; whereas, upon a further addition which increased the value of G by only 11 gm. and the calories by 50, the total

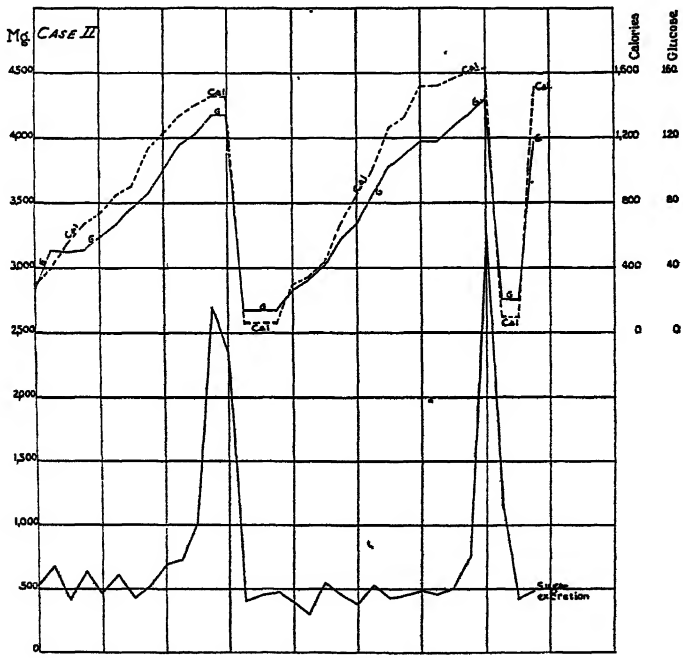

CHART 2.

sugar eliminated was 2,695 mg. Thus, increasing the calories in the diet by some 800 calories and the glucose equivalent by 89 gm. caused little or no increase of the sugar excretion, but the further addition of only 218 calories and the equivalent of 33 gm. glucose caused a critical break in the curve. On the lowest diet the calories were 298; therefore most of the food supply on which the patient was subsisting was then coming from the tissues. The highest diet barely approached maintenance requirements. Accordingly, the figures for the diet never represented the total glucose supply from all sources, and the curve of increase of the latter is not known. Yet the experiment shows a sudden transition from the normal

to the abnormal in respect to the sugar excretion. The diet was then reduced to 64 calories with G for the diet itself at 14; and the experiment was repeated with the same result. There was almost no variation in the total sugar eliminated per day during the time that the diet was increased from 14 G and 64 calories to 135 G and 1,601 calories; whereupon, a subsequent addition of food equivalent to only 9 gm. glucose and 36 calories, which brought G for the total diet to 144 gm. and the calorie value to 1,637, resulted in a sugar excretion of 3,268 mg. for that day. It was quite evident that the curve representing the sugar eliminated in the urine by

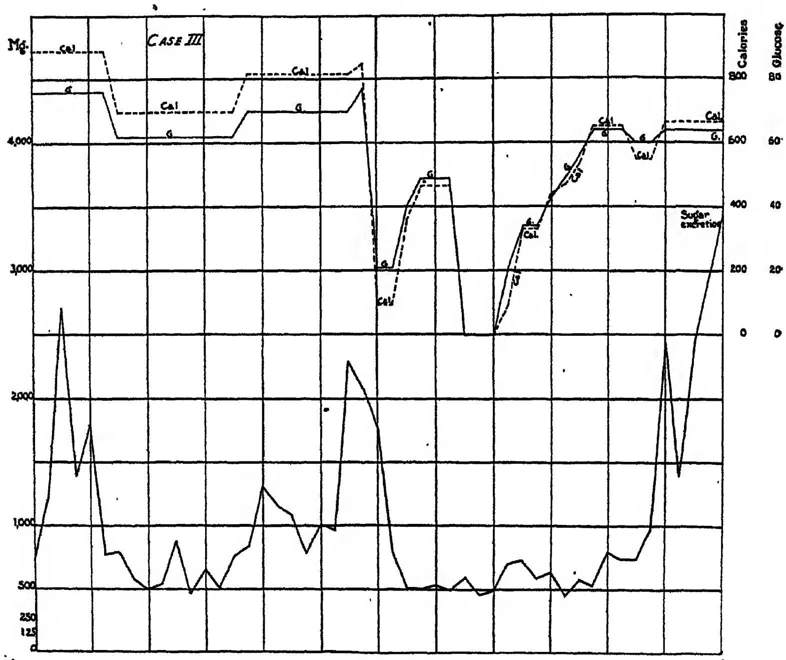

CHART 3.

days, did not follow the curve representing the gradual rise in the caloric or glucose equivalents of the diet. It remained more or less constant until the glucose equivalent of the diet reached a certain value, when there was a sudden upward break in the curve of sugar excretion. This upward break represented an absolute acceleration of the sugar excretion out of proportion to that which had resulted from all earlier additions to the diet. It also represented an even greater increase of the percentage excretion of the last increments to the diet.

Case 3.—(Mrs. F.) A young woman 24 years of age. Weight 36 kilos. Her glucose-using power was very low. On an 888 calorie diet (with

G = 76 gm.) she excreted 725 mg. sugar on 1 day, and 2 days later, while still on the same diet, excreted 2,711 mg. On absolute starvation for 3 consecutive days her urinary sugar excretion was 601, 460, and 504 mg. per day. On a 651 calorie diet, with G = 64 gm., the urinary sugar was 537, 802, and 750 mg., respectively for the 3 days on which the same diet was maintained; whereas, a slight subsequent rearrangement in the diet, increasing its calorie value only to 665, with G as before, resulted in the excretion of 2,440 mg. sugar. Here again, the transition from the "sugar-free" state to one in which the urine contained distinctly

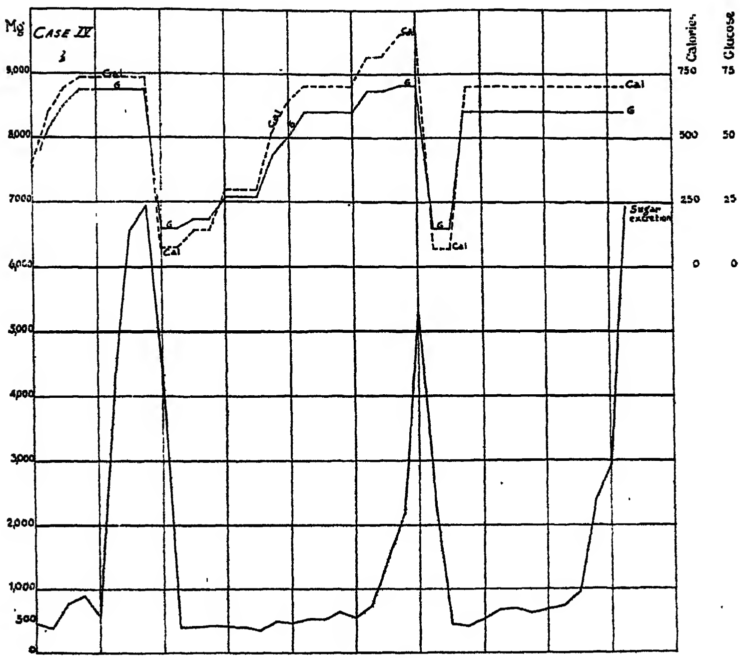

CHART 4.

abnormal quantities of sugar, was a sudden one. [Comment on the cause of the break may be deferred.]

Case 4.—(Mr. M.) A very severe case of diabetes mellitus. The results obtained with this case tend to confirm in a most striking manner those obtained with the above three cases, in that the sugar eliminated in the urine did not increase uniformly with the gradually increasing diet; but that there was a critical point at which a sudden and abnormally high excretion of sugar occurred in the urine.

Case 5.—(Mr. C.) Age 42 years. Weight 52 kilos. This patient's diet was built up gradually from 415 calories with G = 36 gm. to 1,295 calories

with G = 107 gm., while the total sugar eliminated in the urine every 24 hours remained practically constant (except on 1 day following a day of low excretion). In this case the diet at no time reached the point where a real critical acceleration of the sugar excretion occurred. The sugar excreted on the diet of 1,295 calories with G = 107 gm. was no higher than that excreted on the diet of 415 calories, for which G was 36 gm. This patient was neither in caloric nor nitrogenous equilibrium on the diet given, but left the hospital to continue treatment at home so that the chart was interrupted

Case 6.—(Mr. A.) A poorly nourished young man of 25 years. Weight 44.1 kilos. A severe case of diabetes mellitus. This patient came into the hospital with much sugar, acetone, and diacetic acid in the urine, and

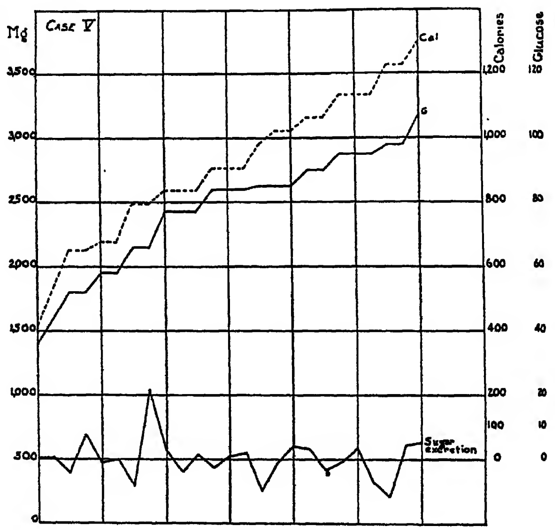

CHART 5.

a marked alkali deficit in the blood. He did not "desugarize" on a diet consisting of 400 gm. of "greens" and 1 liter of broth even after 4 days; and was fed more liberally till the acetone and diacetic acid disappeared. Then, following several dietary manipulations, his urine no longer gave a qualitative test for sugar. On a diet of 953 calories, for which $G = 88$ gm., the total sugar elimination was between 400 and 500 mg. per day; and on a diet of 1,837 calories, with $G = 107$ gm., the sugar excreted in the urine was only 330, 486, and 333 mg.

While the total quantity of sugar eliminated per day on a diet of 64 calories, with G at 14 gm. was practically the same as that eliminated on a diet of 1,993 calories (G = 114.7), it jumped suddenly to 1,485 and 9,800 mg. respectively when the diet was increased to contain 2,080 calories and the

equivalent of 121 gm. glucose. In this case observations were made of the relative effects of a high fat, high carbohydrate, low protein diet; and a high fat, high protein, low carbohydrate diet, each having the same value for *G*. These diets sufficed for maintenance. When the *G* of the diet was kept below a certain value, there was no significant change in the total urinary sugar excreted from day to day, regardless of changes in the relative quantities of carbohydrate and protein. In this case working laterally with maintenance diets, endogenous factors were reduced to a minimum.

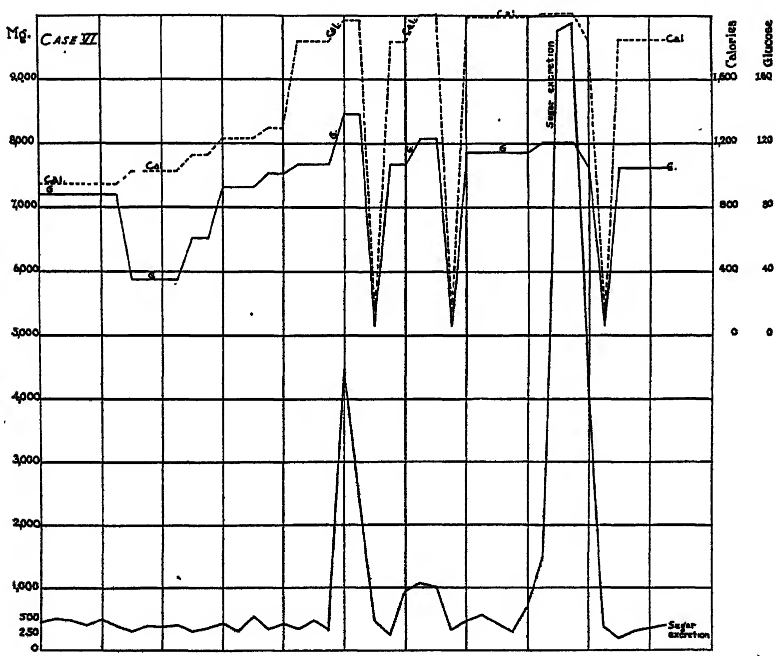

CHART 6.

Case 7.—(Mrs. L.) Age 31 years. Weight 40 kilos. The experiment was begun with the patient on a diet of 830 calories, with $G = 49$. In this case the additions to the diet were made uniform. She was first kept on a constant diet of 25 gm. carbohydrate, 30 gm. protein, and 67 gm. fat, consisting of 400 cc. 5 per cent vegetables, 24 cellulose muffins, 3 eggs, 15 gm. butter, 100 cc. cream, 30 gm. bacon, and 10 gm. rice. For 11 days the sugar excretion was charted while the diet remained the same. During this period the excretion was not at first constant but it became so for the last 5 days (average 562 mg.). Thereafter additions were made always in the form of cream containing 16 per cent fat, with no other changes. The first addition consisted of 50 cc. cream. 3 days later, 25 cc. cream were

added and thereafter 25 cc. more each 4th day until six additions had been made, then 25 cc. cream every other day. In this case each fresh addition to the diet tended to cause a slight increase of the sugar excretion for that day followed by a return to the former level on the following day. These fluctuations were most marked early in the experiment and grew less as time passed. The average excretion was very constant at 600 to 700 mg. While no change took place in the total quantity of sugar eliminated per day (500 to 1,000 mg.), during the time that the glucose equivalent of the diet rose from 49 gm. to 70.3 *G* and the calories from 830 to 1,270, there was a sharp break in the sugar eliminated with the next addition of 25 cc. cream. With the diet aggregating 1,270 calories with $G = 70.3$ gm. the excretion ran 942 and 1,420 mg. on 2 successive days. The next addition was followed by excretions of 1,504 and 1,906 mg. Another addition was

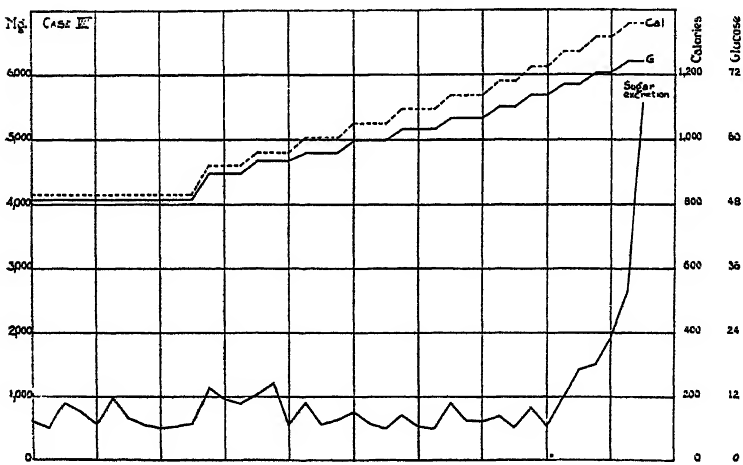

CHART 7.

then made and the excretion rose to 2,650, 5,568, and 6,300 mg. In this case it will be noted that when the total glucose equivalent of the diet was 68 gm. the excretion was still only 825 and 529 mg. on 2 days. The subsequent addition of food equivalent to 6.3 gm. glucose led to the excretion of approximately this quantity of glucose over and above the former average. There was, in short, a virtually complete excretion of all the glucose supplied in excess of a certain limit.

CONCLUSION.

Study of the curves obtained leads to the conclusion that individuals with severe diabetes, when brought into the non-diabetic status (or, as it is sometimes called, the "sugar-free" state) by

fasting or other more suitable adjustments of the diet, may then excrete small quantities of sugar not greater than those excreted by normal individuals under parallel conditions. The quantities in this series averaged between 10 and 15 mg. per kilo per day. As the diet is gradually increased stepwise at 1 to 4 day intervals, there is at first little or no *permanent* increase of the sugar excretion. The sugar excreted has remained entirely unaffected; or it has shown a definite but temporary acceleration with each new addition to the diet to be followed by a restoration of the former level; or it has shown a slight rising tendency from the start. But in any case the total permanent increase of the sugar excretion has remained slight or even unrecognizable until the total glucose equivalent of the diet has risen above a certain limit (which varies with the individual). Once this limit has been passed, further additions to the diet lead to rapid—even sudden—accelerations of the sugar excretions, out of proportion to any which have occurred before. The curve may then bend rapidly upward or show a true critical break.

This observation is in harmony with the well known conception of a clearly definable "tolerance limit" for glucose in diabetes; and that an "abnormal" sugar excretion may develop with critical suddenness when this limit is overstepped.

STUDIES ON PROTEINOGENOUS AMINES.

XII. THE PRODUCTION OF HISTAMINE AND OTHER IMIDAZOLES FROM HISTIDINE BY THE ACTION OF MICROORGANISMS.

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CONTENTS.

INTRODUCTION.

In a series of papers published in 1919[1] we communicated a method for the microchemical colorimetric estimation of imidazole derivatives and for the quantitative separation of histamine from histidine. These methods enabled us to study the metabolism of histidine under various conditions. Experiments

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497, 521, 539.

with a colon bacillus, isolated from a case of cystitis, gave results that led us to the following conclusions:

1. When the colon bacillus is allowed to metabolize histidine, either alone or in the presence of nitrates or ammonium salts, histamine is not formed.

2. In a medium containing histidine and glycerol, but no nitrates or ammonium salts, histamine is not formed. In this case imidazole propionic acid appears to be formed; but only when the bacillus is forced to grow anaerobically.

3. In a medium containing histidine, glycerol, or glucose and a source of nitrogen, either KNO_3, NH_4Cl, or both, about 50 per cent of the histidine is converted into histamine in the course of 2 weeks when oxygen is present. In the absence of atmospheric oxygen, this and all the other metabolic activities of the bacillus are greatly reduced, probably because the colon bacillus is an aerobic organism by preference.

4. The production of histamine is always coincident with the production of a medium that is distinctly acid. We believe that the histamine is formed by the bacillus to neutralize the excess of acidity that is simultaneously produced from the glycerol.

5. Contrary to the statement sometimes given in text-books and in the literature that carbohydrates prevent the formation of histamine from histidine, we have found that histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose.

Having established the above facts for one particular colon bacillus, we were then led to a consideration of the following queries:

1. Are all strains of colon bacilli capable of decarboxylating histidine in our standard medium?

2. Are other organisms capable of converting histidine into histamine under identical conditions?

3. How does the addition of amino-acids or peptones to our standard medium containing histidine, influence the production of histamine?

Procedure.[2]

In every case the same number of microorganisms—nine billion[3]—was introduced into 200 cc. of an autoclaved medium

[2] For a detailed description of the method see Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539–555.

[3] The method employed in counting the organisms was that of Breed and Brew (Breed, R. S., and Brew, J. D., *New York Agric. Exp. Station, Techn. Bull. 49*, 1916).

having the following composition: 0.2000 gm. histidine dichloride, 0.2000 gm. ammonium chloride, 0.1000 gm. potassium nitrate, 0.4000 gm. potassium dihydrogen phosphate, 0.800 gm. sodium chloride, 0.0200 gm. sodium sulfate (anhydrous), 0.4000 gm. sodium bicarbonate, 0.0100 gm. calcium chloride (anhydrous), and 4.00 cc. glycerol dissolved in sufficient distilled water to give a final volume of 200 cc.

The inoculated flasks were then incubated at 37° for 14 days, unless otherwise specified, after which the mixture was forced through a Berkefeld filter. The hydrogen ion concentration of the filtrate was determined colorimetrically (see below for details). The filter was then carefully washed with at least 200 cc. of water. Concentrated sulfuric acid (1.0 cc.) was added to the combined filtrate which was then freed from water by evaporation in a glass dish on the water bath. The syrupy residue was transferred, with distilled water, to a 25 cc. precision cylinder and diluted to exactly 25 cc. Of this test liquid 10 cc. were transferred to a 35 cc. glass-stoppered bottle, treated with 3 gm. of solid sodium hydroxide, and extracted six times with amyl alcohol using 20 cc. for each extraction. This divides the material into two fractions, the amyl alcohol extract, which may contain histamine and methyl imidazole and which we refer to as the histamine fraction, and the alkaline aqueous liquid which contains histidine and may contain imidazole acetic, propionic, lactic, and acrylic acids.

The combined amyl alcohol extracts were extracted with normal sulfuric acid, which removes the imidazoles. The acid extracts were nearly neutralized with 5 N NaOH and the resulting liquid was diluted to exactly 100 cc. The amount of histamine present was then determined colorimetrically by means of the well known reaction that occurs between *p*-phenyldiazonium sulfonate and imidazole derivatives in a solution rendered alkaline with sodium carbonate.[1] When the presence of histamine was indicated by the colorimetric determination, an amino nitrogen determination was also carried out on this fraction. The values obtained by these methods check closely when histidine is the only amino-acid present in the original medium. In other cases the amino nitrogen values are invariably high. To be certain that histamine was the only imidazole present, a methyl imidazole

determination was also carried out. We have never encountered methyl imidazole as a product of the bacterial decomposition of histidine. The presence of histamine was, moreover, qualitatively verified by means of physiological methods.

The histidine fraction was transferred to a 25 cc. graduated precision cylinder with water and 7 cc. of 37 per cent HCl. The cooled acid liquid was then diluted to 25 cc. Portions of this liquid were than tested for histidine, colorimetrically and by means of a Van Slyke amino nitrogen determination. When the values obtained check closely, histidine is probably the only imidazole present. If the color obtained is too red, and the colorimetric determination indicates the presence of considerably more imidazole than can be accounted for as histidine by the amino nitrogen method, the excess color is probably due to imidazole acetic, propionic, lactic, or acrylic acids. Which of these acids is present cannot be determined without an isolation experiment. We have made no effort to isolate these acids but have calculated the excess imidazole value as imidazole propionic acid. When the amino nitrogen determination indicates the presence of considerably more histidine than can be accounted for colorimetrically, we are confronted with two possibilities:

1. Some of the introduced ammonia may have been converted into a carboxylated, alkali-stable, primary amino compound—possibly an amino-acid—which would give off nitrogen with nitrous acid; or

2. Some of the histidine may have suffered a rupture of the imidazole ring with the liberation of free amino groups.

Up to the present time we have brought no absolute proof that either of these is the correct explanation for the facts. At present we are inclined to believe that the second of these possibilities is the most probable and our reason for this belief is outlined in the following pages.

Determination of the Hydrogen Ion Concentration.

In our earlier work we employed the set of standard phenolsulfonephthalein tubes furnished by Hynson, Westcott and Dunning to determine the pH of our media after incubation. This set is inadequate for work of this kind because it is useful only

within the narrow limits of pH 6.6 to 8.6. Most of our final media were too strongly acid to fall within the range of this indicator. We have, therefore, prepared a series of standard tubes with a lower pH value of 1.2 by following the method of Clark and Lubs.[4] The indicators selected were phenol red, range 8.6 to 6.6; brom-cresol purple, range 6.8 to 5.2; methyl red, range 6.0 to 4.4; brom-phenol blue, range 4.6 to 3.0; and thymol blue, range 2.8 to 1.2.

We have found that the buffer solutions containing the sulfonephthalein derivatives can be kept for at least 2 years, and hence probably indefinitely, in a sealed Pyrex glass tube after preservation with thymol. It is possible, therefore, to prepare a permanent set of tubes, similar to those distributed by various concerns, for the phenol red range, for all of the sulfonephthalein derivatives. Methyl red, which is not a sulfonephthalein derivative, deteriorates so rapidly that a set of permanent tubes containing this indicator cannot be prepared in the usual way. We have found, however, that the colors produced with methyl red can be imitated by means of an aqueous solution containing mixtures of Congo red and methyl orange. These thymol-preserved aqueous solutions containing Congo red and methyl orange can then be sealed up and kept in Pyrex glass tubes similar to those used for the other standard sets. They seem to keep indefinitely.

The determinations were carried out as follows. A drop of the filtrate to be tested was transferred to a porcelain test plate and mixed with 1 drop of indicator. This procedure was repeated until a color was obtained that was within the range of one of the indicators. Then 1.0 cc. of the filtrate to be tested was mixed with 0.10 cc. of the proper indicator in a Pyrex test-tube and the color compared with that of the standard tubes. The inherent color of the filtrates was never sufficiently intense to have any effect upon the accuracy of the determination.

Method and Table for the Estimation of Small Amounts of Imidazole Lactic Acid.

In our previous papers[1] we reported tables by means of which colorimetric readings could be converted into milligrams of his-

[4] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1.

tidine, histamine, methyl imidazole, and imidazole acetic and propionic acids. It seemed desirable to have a similar table for the estimation of imidazole lactic acid. This substance was, therefore, prepared using the method described by Fränkel.[5] The perfectly white solid obtained by this method, after two recrystallizations from water, had the following properties.

1. Melting point 217°.
2. Chlorine—none.
3. Residue on ignition—none.
4. Ammonia—none.
5. Amino nitrogen (Van Slyke method)—none.
6. The solid—0.1000 gm.—was dissolved in 10 cc. of 0.10 N NaOH and allowed to react for 1 hour at room temperature. The excess of alkali was determined by titration with 0.10 N HCl, using phenolphthalein as indicator. The first change in the indicator was obtained when 4.3 cc. of the 0.10 N acid had been added, 4.5 cc. of the acid being required to give a colorless solution. The indefinite end-point obtained is exactly what one would expect of a substance having a fairly strong acid group and a feebly basic group. The 5.7 cc. of 0.1 N NaOH used for 0.1000 gm. of substance agrees very well with the 5.74 cc. demanded by theory for $C_6H_8N_2O_3 \cdot H_2O$. We therefore considered the substance to be 100 per cent pure.

A stock solution was prepared by dissolving 0.5000 gm. of the solid in 28.7 cc. of 0.10 N HCl and diluting with water to 50 cc. From this the standard test solution was prepared by diluting 1 cc. to 100 cc. in a volumetric flask. The tabular values were then obtained by mixing different amounts of this standard solution with the alkaline *p*-phenyldiazonium sulfonate reagent as previously described.[1] The color produced was then compared in a Duboscq colorimeter with a standard solution of Congo red.[6]

The color produced matches that of the Congo red solution perfectly. A color of maximum intensity is obtained within 3 to 5 minutes and it is stable for from 5 to 10 minutes during which time an accurate comparison can easily be made. With

[5] Fränkel, S., *Monatsh. Chem.*, 1903, xxiv, 229.

[6] To prepare the Congo red solution, vacuum-dried Grubler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volume flask. Water is then added to the mark. This is the stock solution which keeps indefinitely. From it the standard indicator solution is prepared by diluting 1.00 cc. with distilled water to 500 cc. in a volume flask.

TABLE I.

Estimation of Small Amounts of Imidazole Lactic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole lactic acid ($C_6H_8N_2O_3 \cdot H_2O$) in the test cylinder. (Total volume 8 cc.) Test cylinder set at 20 mm.
mm.	*gm.*
1.0	0.000001
2.0	0.000002
3.0	0.000003
4.1	0.000004
5.1	0.000005
6.1	0.000006
7.1	0.000007
8.1	0.000008
9.2	0.000009
10.2	0.000010
11.2	0.000011
12.2	0.000012
13.2	0.000013
14.3	0.000014
15.3	0.000015
16.3	0.000016
17.3	0.000017
18.3	0.000018
19.4	0.000019
20.4	0.000020
21.4	0.000021
22.4	0.000022
23.4	0.000023
24.5	0.000024
25.5	0.000025
26.5	0.000026
27.5	0.000027
28.5	0.000028
29.6	0.000029
30.6	0.000030
31.6	0.000031
32.6	0.000032
33.6	0.000033
34.7	0.000034
35.7	0.000035
36.7	0.000036
37.7	0.000037

TABLE I—*Concluded.*

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole lactic acid ($C_6H_8N_2O_3.H_2O$) in the test cylinder. (Total volume 8 cc.) Test cylinder set at 20 mm.
mm.	*gm.*
38.7	0.000038
39.8	0.000039
40.8	0.000040
41.8	0.000041
42.8	0.000042
43.8	0.000043
44.9	0.000044
45.9	0 000045
46.9	0.000046
47.9	0.000047
48.9	0.000048
50.0	0.000049
51.0	0.000050

quantities of this acid in excess of 0.000025 gm., the color produced is too intense to enable one to make an accurate comparison. In such cases the test cylinder was set at 10 instead of at 20. The reading obtained was then multiplied by two before subtracting the correction blank, which is 0.30 mm. as in the case of the other imidazoles.[7]

PART I.

On The Products Formed From Histidine by the Action of Bacillus coli.

These four organisms (*Bacillus coli communis, Bacillus coli communior, Bacillus lactis aerogenes,* and *Bacillus acidi lactici*) comprise the colon group (in the narrower sense) according to the classification of the American Public Health Association. This differentiation is based upon the different behavior of these four organisms toward dextrose, lactose, dulcitol, and saccharose and is represented as follows:

[7] This correction blank represents the amount of color that is autogenously produced by the reagent even when imidazoles are absent. See foot-note 1.

Bacillus coli Group.

Dextrose +
Lactose +

- Dulcitol +
  B. communior
  B. communis
  - Saccharose +
    B. communior
  - Saccharose −
    B. communis
- Dulcitol −
  B. aerogenes
  B. acidi lactici
  - Saccharose +
    B. aerogenes
  - Saccharose −
    B. acidi lactici

The behavior of the organisms investigated by us is summarized in Table II. We have included a large number of tests that were not required for differentiation purposes because we hoped to find a correlation between these reactions and the production of histamine. Such a relationship seems, however, not to exist. The organisms Coli K, H, B, 88, 51, 80, 84, 90, 52, 74, and Schwartz were isolated from human feces and investigated as soon as they had been obtained in pure culture. The other strains were stock cultures obtained from a variety of sources. Some of these strains have been growing on artificial media for years.

Bacillus coli communior.

Of the organisms investigated, eight belonged to this group. Their behavior on our synthetic medium is summarized in Table III.

Coli P-3-19.—This organism did not rupture the imidazole ring to an appreciable extent. Of the histidine originally introduced, 29.7 per cent was converted into histamine and 59 per cent was recovered unchanged. Imidazoles other than histamine were not formed. Of the ammonia originally introduced, 42 per cent was removed by the microorganisms.

Coli K (*red*).—There is a sufficient discrepancy between the amino nitrogen and color values for both the histidine and the histamine fractions to suggest a slight rupture of the imidazole ring with the production of primary amino groups. Of the histidine originally introduced, 37.6 per cent was converted into histamine and 50 per cent was recovered unchanged. Imidazoles other than histamine were not formed. Of the ammonia originally introduced, 9.7 per cent was removed by the bacilli.

TABLE II.

Name of strain.	Mobility.	Gram stain.	Colony on endo.	Gelatin slab.	Milk (coagulation of).	Dextrose.	Lactose.	Saccharose.	Galactose.	Levulose.	Maltose.	Raffinose.	Mannitol.	Mannose.	Arabinose.	Xylose.	Dulcitol.	Inulin.	Salicin.	Indole.	Voges-Proskauer.	Histamine production.	Classification.
Coli P-3-19	−	−	Red.	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	Bacillus communior.
" K (red)	−	−	"	−	+	+	+	+				+	+				+					+	
" Y	−	−	"	−	+	+	+	+									+					+	
" Jd	−	−	"	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+		−	
" Lac. Aer.	−	−	"	−	+	+	+	+									+					−	
" 88	−	−	"	−	+	+	+	+				+	+				+					−	
" 51	−	−	"	−	+	+	+	+				−	+				+					−	
" H (white)	−	−	White.	−	+	+	+	+									+						
" cystitis	−	−	Red.	−	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	−	+	Bacillus communis.
" Wk	−	−	"	−	+	+	+	−	+	+	+	−	+	+	−	−	+	−	−	+	−	−	
" Hm	−	−	"	−	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	−	−	
" Cs	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	+	+	−	−	+	−	−	
" K (white)	−	−	White.	−	+	+	+	−				−	+				+					−	
" bovis No. 3	−	−	Red.	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	Bacillus aerogenes.
" " " 4	−	−	"	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+		−	
" 80	−	−	"	−	+	+	+	+				+	+				−					−	
" 84	−	−	"	−	+	+	+	+				+	+				−					−	
" 90	−	−	"	−	+	+	+	+				+	+				−					−	

Coli B	—	—	Red.	—	+	+	+	—				—	+				—					—	
" H	—	—	"	—	+	+	+	—									—					—	
" P-1-19	—	—	"	—	+	+	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	
" P-2-19	—	—	"	—	+	+	+	—	+	+	+	—	+	+	+	+	—	—	—	+	—	—	
" P-4-19	—	—	"	—	+	+	+	—	+	+	+	—	+	+	+	—	—	—	—	+	—	—	
" P-5-19	—	—	"	—	+	+	+	—	+	+	+	—	+	+	+	—	—	—	—	+	—	+	*Bacillus acidi lactici.*
" P-6-19	—	—	"	—	+	+	+	—	+	+	+	—	+	+	+	—	—	—	—	+	—	—	
" I (K)	—	—	"	—	+	+	+	—	+	+	+	—	+	+	+	+	—	—	—	+	—	—	
" Schwartz	—	—	"	—	+	+	+	—				+	—				—						
" 52	—	—	"	—	+	+	+	—				—	+				—					—	
" 51 (white)	—	—	White.	—	+	+	+	—				—	+				—					—	
" 74	—	—	Red.	—	+	+	+	—				—	+				—					—	

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli P-3-19.	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Match perfect. 96%	0.10 cc. = 5.9 mm. 0.20 " = 11.8 " Match good. 59%	1.18 cc. N_2 at 22° and 745 mm. 0.1327 gm. histidine dichloride. 66.3%
Coli K (red).	0.10 cc. = 10.8 mm. 0.20 " = 21.6 " Match perfect. 108%	0.10 cc. = 5.0 mm. 0.20 " = 10.0 " Match perfect. 50%	1.14 cc. N_2 at 26° and 750 mm. 0.1264 gm. histidine dichloride. 63.2%
Coli Y, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.63 cc. N_2 at 27.5° and 744 mm. 0.1795 gm. histidine dichloride. 89.7%
30 days.	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Match perfect. 74%	0.10 cc. = 6.6 mm. 0.20 " = 13.2 " Match good. 66%	2.46 cc. N_2 at 24° and 748 mm. 0.275 gm. histidine dichloride. 137.5%
Coli Jd.	0.10 cc. = 10.1 mm. 0.20 " = 20.2 " Match perfect. 101%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.55 cc. N_2 at 22° and 743 mm. 0.1738 gm. histidine dichloride. 86.9%
Coli Lac. Aer., 7 days.	0.10 cc. = 8.7 mm. 0.20 " = 17.4 " Match perfect. 87%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. 75%	1.37 cc. N_2 at 16° and 750 mm. 0.1597 gm. histidine dichloride. 79.8%
14 days.	0.10 cc. = 5.2 mm. 0.20 " = 10.4 " Match perfect. 52%	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match good. 45%	1.81 cc. N_2 at 23° and 748 mm. 0.2032 gm. histidine dichloride. 101.6%

* Colors matched against the (CR-MO) standard.

coli communior.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
0.05 cc. = 7.2 mm. 0.10 " = 14.4 " Color develops like that of histamine.	0.048 gm. of histamine in entire test solution. 29.7% of histamine present.	0.46 cc. N_2 at 22° and 748 mm. 0.042 gm. histamine dichloride. 26%	21.0 Hence the ≎ of 15 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.9
0.05 cc. = 9.1 mm. 0.10 " = 18.2 " Color develops like that of histamine.	0.0607 gm. of histamine in entire test solution. 37.6% of histamine present.	0.9 cc. N_2 at 30° and 748 mm. 0.0785 gm. histamine dichloride. 48.6%	32.5 Hence the ≎ of 3.5 cc. of 0.1 N NH_3 used by the microorganisms.	7.4	5.4
0.50 cc. = 4.8 mm. 1.00 " = 9.6 " Color develops like that of histamine.	0.00128 gm. of histamine in entire test solution. 1.98%		20.0 Hence the ≎ of 16 cc. of 0.1 N NH_3 used by microorganisms.	7.3	6.0
0.20 cc. = 4.0 mm. 0.40 " = 8.0 " Color develops like that of histamine.	0.00668 gm. of histamine dichloride in entire test solution. 4.1%		None. Hence the ≎ of 36 cc. of 0.1 N NH_3 used by microorganisms.	7.3	4.6
None.			28.0 Hence the ≎ of 8 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.6
None.			36.7 Hence the ≎ of 0.7 cc. of 0.1 N NH_3 was produced by microorganisms.	7.3	5.8
None.			38.0 Hence the ≎ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	5.4

TABLE III—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli 88.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.3 mm. 0.20 " = 16.6 " Match perfect. 83%	1.62 cc. N_2 at 27° and 746 mm. 0.1775 gm. histidine dichloride 88.8%
Coli 51.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	1.62 cc. N_2 at 27° and 746 mm. 0.1775 gm. histidine dichloride. 88.8%

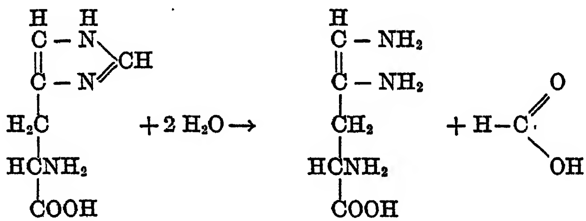
Concluded.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			37.0 Hence the ≎ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.4
None.			33.75 Hence the ≎ of 2.25 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.4	5.6

Coli Y.—During the first 2 weeks of incubation, 8 per cent of the introduced histidine and 44 per cent of the introduced ammonia were removed by the microorganisms. Of the histidine originally introduced, about 2 per cent was converted into histamine. There is no evidence that the imidazole ring was ruptured because the check between the histidine values by amino nitrogen and colorimetric determinations is perfect.

During the next 16 days of incubation an additional 2 per cent of histamine was produced. The liquid was *free from ammonia;* hence 100 per cent of the ammonia originally introduced was removed by the organisms. The most striking observation, however, was the discrepancy between the histidine values obtained by the colorimetric and the amino nitrogen methods.

In a previous paper we called attention to the fact that histidine might be decomposed by microorganisms according to the following structure:



The triamino compound formed, because of its carboxyl group, would form a sodium salt, in the presence of a strong alkali, that should be soluble in water and difficultly soluble in amyl alcohol. In the course of our standard procedure this triamino compound should appear together with histidine in the histidine fraction. Although histidine contains but one primary amino group, the triamino compound contains three such groups; hence each molecule of triamino compound would give three times as much nitrogen, by the Van Slyke method, as a molecule of histidine.

Going back to the table we find that 90 per cent of the introduced histidine was left unchanged at the end of 14 days. After 30 days of incubation, 66 per cent of histidine was present and 2 additional per cent of histamine had been produced. In all, then, 68 of the 90 per cent of histidine left after 14 days of incubation can be accounted for colorimetrically. What became of the 22 per cent of histidine that cannot be accounted for colorimetrically? If we assume that all of this histidine was converted into triamino compound, an amount of N_2 equivalent to 22 times 3 or 66 per cent of histidine would be evolved in an amino nitrogen determination.

If to this we add the nitrogen evolved by the 66 per cent of histidine present in the same liquid, one would expect to obtain an amount of nitrogen equivalent to 132 per cent of histidine which compares very well with the 137.5 per cent actually obtained.

We realize, of course, that an excess of amino nitrogen may not necessarily indicate the presence of a histidine disruption product. It is possible that non-volatile, carboxylated amino compounds, possibly amino-acids, might be synthesized from ammonia and glycerol, and if these were present, the amino nitrogen figure would be high. At present, however, we are inclined to believe that a triamino compound is responsible for the excess amino nitrogen because a quantitative relationship similar to the one given above has been found to hold in five other cases. A quantitative agreement might be obtained once or twice by accident; but it seems hardly reasonable to assume that the accident should occur six times.

The formula of the triamino compound would lead one to believe that the compound might have some physiological activity. A search of the literature revealed the fact that diamino acetylene derivatives have not yet been prepared. We hope to make the preparation and properties of this triamino compound the subject of a subsequent paper.

Finally we can raise the question, *why do certain microorganisms rupture the imidazole ring with the liberation of free amino groups?* Two reasons suggest themselves. This type of nuclear disruption is the most certain way to expose for future use all of the nitrogen and carbon of the molecule. This is also a decomposition that converts a feebly basic substance into one that is strongly basic.

Coli Jd.—This organism did not rupture the imidazole ring. Of the histidine originally introduced, 86 per cent was recovered unchanged. Histamine and other imidazoles were not formed. Of the ammonia originally introduced 22 per cent was removed by the microorganisms.

Coli Lac. Aer.—During the first 7 days of incubation, this rapidly growing organism reduced the histidine concentration of the solution to 75 per cent of its initial value. There is practically no indication that a nuclear rupture occurred. The concentration of ammonia in the solution was *greater* after 7 days of incubation than it was at the outset of the experiment. Some of this ammonia must have been derived either from the disrupted histidine, or from the potassium nitrate.

The second 7 day period of incubation reduced the histidine concentration to 45 per cent of its initial value. Some of this histidine was apparently converted into triamino compound and some into ammonia. Although there is no doubt, in this case, that a carboxylated amino compound was formed from the histidine, quantitative proof for the formation of a triamino compound is lacking because the decomposition continued beyond this stage with the production of ammonia. Histamine and other imidazoles were not formed.

The increase in the ammonia figure might suggest that this organism was unable to utilize ammonia as a source of nitrogen, and that the imidazole ring was ruptured to render nitrogen available. This was, however, not the case because this organism grows splendidly on a medium that contains only inorganic salts, glycerol, and NH_4Cl. On this histidine-free medium the ammonia value drops from 36 to 25 cc. of 0.1 N HCl in the course of 2 weeks. The rupture of the imidazole ring might, therefore, have been resorted to in an attempt to lower the hydrogen ion concentration of the cell protoplasm.

Coli 88.—This organism did not rupture the imidazole ring with the production of appreciable quantities of a carboxylated amino compound; but some of the histidine nitrogen must have been converted into ammonia because the concentration of ammonia at the end of the 14 day incubation period was greater than the initial concentration. Of the histidine originally introduced, 83 per cent was recovered unchanged. Histamine and other imidazoles were not formed.

Coli 51.—The results obtained with this organism were so nearly like those obtained with Coli 88 that a detailed discussion seems superfluous. In this case, however, the ammonia consumption was more rapid than its production so that the initial ammonia value was reduced by 6.2 per cent.

Bacillus coli communis.

Of the organisms investigated, five belong to this group. Their behavior on our synthetic medium is summarized in Table IV.

Coli cystitis.—This is the organism that was used in our earlier work.[1] Since its behavior has been described in detail in our previous articles, it seems unnecessary to do more here than to call attention to the fact that it has lost none of its power of decarboxylation. In this experiment 57 per cent of the histidine originally introduced was converted into histamine as compared to a 50 per cent conversion obtained by us some 2 years ago.

Coli Wk.—This organism ruptured the imidazole ring to a considerable extent, apparently with the formation of a carboxylated amino compound. Quantitative proof for the formation of a triamino compound is, however, lacking in this case. Of the histidine originally introduced 76 per cent was recovered. Histamine and other imidazoles were not formed. Of the

ammonia originally introduced, 8.3 per cent was removed by the microorganisms.

Coli Hm.—This organism seems not to have attacked the histidine at all because 96 per cent of this amino-acid was recovered. The nitrogen requirements were obviously supplied by the ammonia whose final concentration was only 75 per cent of that originally introduced.

Coli Cs.—This organism did not rupture the imidazole ring with the formation of a carboxylated amino compound. Of the histidine originally introduced, 86 per cent was recovered. Histamine or other imidazoles were not formed. Of the ammonia originally introduced, 25 per cent was removed by the microorganisms.

Coli K (White).—This organism ruptured the imidazole ring to the extent of 8 per cent, apparently with the formation of a triamino compound (see under Coli Y). Of the histidine originally introduced, 92 per cent was recovered. If we assume that the 8 per cent of histidine that disappeared in the course of 2 weeks incubation, was quantitatively converted into triamino compound, the total amino nitrogen value of the liquid should have been eight times 3 plus 92 equals 116 per cent, which compares very well with the 113.4 per cent actually obtained. Histamine and other imidazoles were not formed. A small amount of ammonia was produced either from the histidine or from the potassium nitrate.

Bacillus lactis aerogenes.

Of the organisms investigated, five belong to this group. Their behavior on our synthetic medium is summarized in Table V.

Coli bovis 3.—During the first 2 weeks of incubation, this organism reduced the histidine concentration of the solution to 92 per cent of its initial value and removed 17 per cent of the ammonia that was originally introduced. There is no indication that a nuclear rupture occurred. Histamine and other imidazoles were not produced.

After 30 days of incubation, the histidine concentration was reduced to 84 per cent of its initial value. During this period, 2.4 per cent of histidine was converted into histamine. In all, then, 86.4 per cent of histidine can be accounted for colorimetrically, after 30 days of incubation. After 14 days of incubation, 92 per cent of histidine was recovered. If we assume that the 5.6 per cent of histidine that disappeared during the second 16 day period was converted quantitatively into triamino compound, an amino nitrogen value of 5.6 times 3 plus 84 equals 100.8 per cent should have been obtained for the histidine fraction which compares very well with the 103.5 per cent actually obtained. This organism seems, therefore, to have converted 5.6 per cent of histidine into triamino compound. Of the ammonia originally introduced, 28.5 per cent was removed by the microorganisms.

TABLE IV—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli cystitis.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Color developed very rapidly. 112%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. 40%	0.85 cc. N_2 at 20° and 750 mm. 0.097 gm. histidine dichloride. 48.5%
Coli Wk.	0.10 cc. = 8.4 mm. 0.20 " = 16.9 " Match perfect. 84%	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match good. 76%	1.95 cc. N_2 at 22° and 748 mm. 0.220 gm. histidine dichloride. 110%
Coli Hm.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. 103%	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Match perfect. 96%	1.80 cc. N_2 at 20° and 750 mm. 0.206 gm. histidine dichloride. 103%
Coli Cs.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.57 cc. N_2 at 23° and 750 mm. 0.1768 gm. histidine dichloride. 88.4%
Coli K (white).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.2 mm. 0.20 " = 18.3 " Match perfect. 92%	2.07 cc. N_2 at 28° and 750 mm. 0.2268 gm. histidine dichloride. 113.4%

* Colors matched against the (CR–MO) standard.

coli communis.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
0.05 cc. = 13.8 mm. 0.10 " = 27.5 " Color develops like that of histamine.	0.092 gm. of histamine dichloride in the entire test solution. 57% of histamine present.	1.04 cc. N_2 at 20° and 754 mm. 0.0964 gm. histamine dichloride. 59.8%	27 Hence the ≏ of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.4
None.			33 Hence the ≏ of 3 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	6.4
None.			27 Hence the ≏ of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.2
None.			27 Hence the ≏ of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.8
None.			37 Hence the ≏ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.5

TABLE V—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli bovis 3, 14 days.	0.10 cc. = 10.6 mm. 0.20 " = 21.2 " Match perfect. 106%	0.10 cc. = 9.2 mm. 0.20 " = 18.4 " Match perfect. 92%	1.65 cc. N_2 at 27° and 749 mm. 0.182 gm. histidine dichloride. 91%
30 days.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.4 mm. 0.20 " = 16.7 " Match perfect. 84%	1.85 cc. N_2 at 21° and 738 mm. 0.207 gm. histidine dichloride. 103.5%
Coli bovis 4.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. 103%	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	1.93 cc. N_2 at 26° and 745 mm. 0.212 gm. histidine dichloride. 106%
Coli 80.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.97 cc. N_2 at 30° and 747 mm. 0.213 gm. histidine dichloride. 106.5%
Coli 84.	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.79 cc. N_2 at 25° and 751 mm. 0.2000 gm. histidine dichloride. 100%
Coli 90.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.51 cc. N_2 at 21° and 752 mm. 0.172 gm. histidine dichloride. 86%

* Colors matched against the (CR-MO) standard.

lactis aerogenes.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction. Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			29.7 Hence the ≎ of 6.3 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.4
0.40 cc. = 4.7 mm. 0.80 " = 8.4 " Color develops like that of histamine.	0.0039 gm. of histamine dichloride in the entire test solution. 2.4% of histamine present.		25.7 Hence the ≎ of 10.3 cc. of 0.10 N NH_3 used by the microorganisms.	7.3	5.2
None.			36.0 Hence appreciable quantities of NH_3 seem not to have been removed by the microorganisms.	7.4	5.4
None.			29 Hence the ≎ of 7 cc. of 0.1 N NH_3 used by the microorganisms.	7.4	5.4
None.			37 Hence the ≎ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			37.5 Hence the ≎ of 1.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6

Coli bovis 4.—This organism did not grow as well as the other coli and it seems not to have attacked appreciable quantities of either the histidine or the ammonia.

Coli 80.—This colon bacillus ruptured the imidazole ring with the formation of carboxylated amino compounds. Of the histidine originally introduced, 80 per cent was recovered. Histamine and other imidazoles were not formed. The ammonia concentration was reduced by 19.5 per cent.

Coli 84.—A nuclear rupture with the formation of carboxylated amino compounds is again indicated in this case. Of the histidine originally introduced, 86 per cent was recovered. Some ammonia was produced either from the disrupted histidine or from the potassium nitrate. Histamine or other imidazoles were not formed.

Coli 90.—The liquid finally obtained in this case was free from carboxylated amino compounds other than histidine. Of this amino-acid, 84 per cent was recovered unchanged. Some ammonia was produced either from the disrupted histidine or from the potassium nitrate. Histamine or other imidazoles were not formed.

Bacillus acidi lactici.

Of the organisms investigated, twelve belong to this group. Their behavior on our synthetic medium is summarized in Table VI.

Ten of these organisms gave results that are qualitatively identical; so they can be discussed collectively. Only Coli P-2–19 and P–5–19 require special consideration.

All of the ten similar organisms ruptured the imidazole ring with the production of carboxylated amino compounds. The disruption did not proceed quantitatively to the formation of triamino compound in any of these cases. Histamine and other imidazoles were not formed. Some of the organisms—Coli H, Schwartz, 52, 51 (white), and 74—produced ammonia, and others—Coli P-1-19, P–4–19, P–6–19, and I (K)—removed some from the solution.

Coli P-2-19.—The formation of a carboxylated triamino compound is again quantitatively indicated in this case. Of the histidine originally introduced, 95 per cent was recovered. If we assume that the 5 per cent of histidine that disappeared was quantitatively converted into a triamino compound, an amino nitrogen value of five times 3 plus 95 or 110 per cent should have been obtained which agrees almost exactly with the 110.2 per cent actually obtained. Histamine and other imidazoles were not formed. Of the ammonia originally introduced, 57 per cent was removed by the microorganisms.

Coli P-5-19.—This interesting organism converted 14.5 per cent of the histidine originally introduced, into histamine. Since 79 per cent of histidine was recovered unchanged, 93.5 per cent of the original histidine can be accounted for colorimetrically. The amino nitrogen determination on the histidine fraction gave a value of 100.5 per cent calculated as histidine. Of this amino nitrogen only 79 per cent could have been derived from histidine. If we assume that the remaining 21.5 per cent of amino nitrogen was derived from the triamino compound, this would account for 7.17 per cent of histidine; *i.e.*, 21.5 divided by 3. Summing up, then, we have

79.0	per cent as histidine.
14.5	" " " histamine.
7.17	" " " triamino compound.
100.67	" " total recovery.

which is a truly remarkable agreement.

PART II.

The Products Formed from Histidine by the Action of Other Members of the Colon Typhoid Group.

The organisms investigated were *Bacillus enteritidis, Bacillus typhosus, Bacillus paratyphosus* A (3 strains), *Bacillus dysenteriæ* Flexner, *Bacillus dysenteriæ* Morgan, *Bacillus dysenteriæ* Shiga; *Bacillus fæcalis alcaligenes* I, and *Bacillus fæcalis alcaligenes III.* The behavior of these organisms on our synthetic medium is summarized in Table VII.

B. enteritidis 228.—This organism grew very well on the liquid medium. There is no evidence of a rupture of the imidazole ring after 7 days of incubation although 20 per cent of the introduced histidine disappeared during this time interval. Of the ammonia originally introduced 32 per cent was removed by the microorganisms. Histamine and other imidazoles were not formed.

The attack on the remaining histidine was so intense, during the second 7 day period, that 40 per cent of that amino-acid disappeared. A nuclear rupture occurred with the formation of some carboxylated amino compound and considerable ammonia. Histamine and other imidazoles were not formed.

B. typhosus.—This organism grew poorly on our medium. Histamine and other imidazoles were not formed. Very little acid was produced. Of the ammonia originally introduced, 80 per cent was recovered. The results on the histidine fraction suggest that a small amount of histidine was deaminized because the colorimetric value for histidine ran higher than the amino nitrogen value. The conversion was too slight, however, to be of particular significance.

TABLE VI—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli B.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.2 mm. 0.20 " = 18.4 " Match perfect. 92%	1.90 cc. N_2 at 28° and 747 mm. 0.208 gm. histidine dichloride. 104%
Coli H.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.80 cc. N_2 at 30° and 747 mm. 0.1945 gm. histidine dichloride. 97.2%
Coli P-1-19.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.1 mm. 0.20 " = 18.2 " Match perfect. 91%	1.70 cc. N_2 at 21° and 744 mm. 0.192 gm. histidine dichloride. 96%
Coli P-2-19.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	1.99 cc. N_2 at 25° and 745 mm. 0.2204 gm. histidine dichloride. 110.2%
Coli P-4-19.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.4 mm. 0.20 " = 16.7 " Match perfect. 84%	1.85 cc. N_2 at 22° and 747 mm. 0.209 gm. histidine dichloride. 104.5%
Coli P-5-19.	0.10 cc. = 9.8 mm. 0.20 " = 19.4 " Match perfect. 98%	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. 79%	1.77 cc. N_2 at 20° and 745 mm. 0.201 gm. histidine dichloride. 100.5%
Coli P-6-19.	0.10 cc. = 9.4 mm. 0.20 " = 18.9 " Match perfect. 94%	0.10 cc. = 9.4 mm. 0.20 " = 18.8 " Match perfect. 94%	1.81 cc. N_2 at 21° and 746 mm. 0.205 gm. histidine dichloride. 102.5%

* Colors matched against the (CR-MO) standard.

acidi lactici.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.				7.4	5.8
None.			38 Hence the ≎ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			31 Hence the ≎ of 5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.7
None.			15.5 Hence the ≎ of 20.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.7
None.			32.5 Hence the ≎ of 3.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.9
0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Color develops like that of histamine.	0.0235 of histamine dichloride in the entire test solution. 14.5% of histamine present.	0.35 cc. N_2 at 22° and 744 mm. 0.0317 gm. histamine dichloride. 19.6%	35.5 Hence the ≎ of 0.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	6.2
None.			27 Hence the ≎ of 9 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	6.0

TABLE VI.

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli 1 (K).	0.10 cc. = 9.0 mm. 0.20 " = 18.1 " Match perfect. 90%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.71 cc. N_2 at 22.5° and 745 mm. 0.1925 gm. histidine dichloride. 96.2%
Coli Schwartz.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. 89%	1.89 cc. N_2 at 28° and 749 mm. 0.207 gm. histidine dichloride. 103.5%
Coli 52.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.87 cc. N_2 at 27° and 746 mm. 0.205 gm. histidine dichloride. 102.5%
Coli 51 (white).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	1.89 cc. N_2 at 28° and 749 mm. 0.207 gm. histidine dichloride. 103.5%
Coli 74.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.8 mm. 0.20 " = 17.6 " Match perfect. 88%	1.83 cc. N_2 at 28° and 747 mm. 0.200 gm. histidine dichloride. 100%

Concluded.

Color value of histamine fraction.	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction. Before incubation.	Reaction. After incubation.
			cc.	*pH*	*pH*
None.			32.5 Hence the ≈ of 3.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.3	5.5
None.			38.5 Hence the ≈ of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			39 Hence the ≈ of 3 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.4
None.			38 Hence the ≈ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	7.0
None.			37 Hence the ≈ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.5

TABLE VII—*Colon*

Name of strain.	Total color value of test solution of histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus enteritidis 228, 7 days.	0.10 cc. = 8.6 mm. 0.20 " = 17.1 " Match perfect. 86%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.46 cc. N_2 at 29° and 746 mm. 0.1584 gm. histidine dichloride. 79.2%
14 days.	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match poor. Color too yellow. 40%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	1.25 cc. N_2 at 23° and 745 mm. 0.140 gm. histidine dichloride. 70%
Bacillus typhosus.	0.10 cc. = 9.6 mm. 0.20 " = 19.1 " Match perfect. 96%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.35 cc. N_2 at 21° and 743 mm. 0.152 gm. histidine dichloride. 76%
Bacillus paratyphosus A 3.	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	0.10 cc. = 8.3 mm. 0.20 " = 16.7 " Match perfect. 83%	1.47 cc. N_2 at 20° and 738 mm. 0.1655 gm. histidine dichloride. 82.7%
Bacillus paratyphosus A 4, 7 days.	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	1.73 cc. N_2 at 28° and 746 mm. 0.1886 gm. histidine dichloride. 94.3%
14 days.	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	1.95 cc. N_2 at 24° and 753 mm. 0.2195 gm. histidine dichloride. 109.7%
Bacillus paratyphosus A (K).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine. 100%	0.95 cc. N_2 at 24° and 753 mm. 0.107 gm. histidine dichloride. 53.5%

* Colors matched against the (CR–MO) standard.

Typhoid Group.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	24.5 Hence the $\approx$ of 11.5 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	6.0
None.	None.	None.	39.5 Hence the $\approx$ of 15 cc. of 0.1 N NH_3 was produced during the second 7 day period.	7.3	5.2
	None.	None.	29 Hence the $\approx$ of 7 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	6.6
None.	None.	None.	31.5 Hence the $\approx$ of 4.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
None.	None.	None.	39 Hence the $\approx$ of 3 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	6.4
None.	None.	None.	38 Hence the $\approx$ of 1 cc. of 0.1 N NH_3 was removed during the second 7 day period.	7.3	5.4
0.034 gm. 27.7%	None.	None.	30.5 Hence the $\approx$ of 5.5 cc. of 0.10 N NH_3 was removed by the microorganisms.	7.3	6.8

TABLE VII.

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus dysenteriæ Flexner.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine. 100%	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Color too pink and develops too rapidly for histamine. 96%	0.91 cc. N_2 at 22° and 745 mm. 0.1025 gm. histidine dichloride. 51.2%
Bacillus dysenteriæ Morgan.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect, color develops rapidly. 95%	0.10 cc. = 7.8 mm. 0.20 " = 15.6 " Match perfect, color develops rapidly. 78%	1.15 cc. N_2 at 22° and 743 mm. 0.129 gm. histidine dichloride. 65%
Bacillus dysenteriæ Shiga.	0.10 cc. = 11.3 mm. 0.20 " = 22.6 " Color too pink and develops too rapidly for histamine. 113%	0.10 cc. = 9.3 mm. 0.20 " = 18.5 " Color too pink and develops too rapidly for histamine. 93%	1.24 cc. N_2 at 19° and 748 mm. 0.1423 gm. histidine dichloride. 71.1%
Bacillus fæcalis alcaligenes I.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Color slightly too pink. 97%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Color slightly too pink for histidine. 70%	0.80 cc. N_2 at 23° and 749 mm. 0.090 gm. histidine dichloride. 45%
Bacillus fæcalis alcaligenes III, 3 days.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match good. 90%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Match perfect. 70%	1.30 cc. N_2 at 23° and 750 mm. 0.1464 gm. histidine dichloride. 73.2%
14 days.	0.10 cc. = 9.2 mm. 0.20 " = 18.5 " Match perfect. 92%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Match perfect. 70%	1.17 cc. N_2 at 23° and 749 mm. 0.1317 gm. histidine dichloride. 66%

Concluded.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
0.033 gm. 27.1%	None.	None.	34.5 Hence the ≈ of 1.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.010 gm. 8.1%	None.	None.	35.5 Hence the ≈ of 0.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.016 gm. 13%	None.	None.	33 Hence the ≈ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.018 gm. 14.65%	None.	None.	32.5 Hence the ≈ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4
None.	None.	None.	32.5 Hence the ≈ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4
None.	None.	None.	33 Hence the ≈ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.6

B. paratyphosus A 3.—This organism grew very poorly on our medium, apparently because an available carbon source was lacking. Both the glycerol and the histidine remained largely unattacked. Histamine, other imidazoles, and acid were not formed. Of the ammonia originally introduced, 13 per cent was removed by the bacilli.

B. paratyphosus A 4.—This organism grew far better than *B. paratyphosus* A 3. Some of the glycerol was metabolized with the production of acid. The imidazole ring was ruptured to a considerable extent with the formation of carboxylated amino compounds and ammonia. Histamine and other imidazoles were not formed.

B. paratyphosus A (K).—This organism deaminized histidine with the formation of imidazole acetic, propionic, lactic, or acrylic acids because the color produced by the histidine fraction was far too red for histidine and the amino nitrogen value was much lower than the colorimetric value. If we assume that *the amino nitrogen was derived exclusively from histidine*, the presence of a maximum of 53.5 per cent of that substance is indicated. Colorimetrically this would give a reading of 5.35 mm. (CR − MO) for 0.10 cc. of the diluted test solution. The reading obtained was 10.0 mm. (CR − MO) for 0.10 cc. The difference between these two readings—4.65 mm. (CR − MO) or 5.12 mm. (CR)[8]—must have been due to either imidazole acetic, propionic, lactic, or acrylic acids. Which of these acids was present was not determined; but the excess color value was calculated as imidazole propionic acid using the table that was previously published for that substance. The calculations show that 0.034 gm. of imidazole propionic acid was present; hence 27.7 per cent of the histidine originally introduced was deaminized.

This organism grew poorly, used glycerol to only a small extent, produced very little acid, and removed the equivalent of 16 per cent of the ammonia originally introduced. Histamine was not formed.

B. dysenteriæ Flexner, Morgan, and Shiga.—The three classes of dysentery bacilli can most advantageously be discussed together because, although the results are quantitatively somewhat different, they are qualitatively identical. In every case the color obtained with the histidine fraction was too red to have been due only to histidine. The colorimetric values were higher than the amino nitrogen values on this fraction; hence histidine was not the only imidazole present. When the discrepancies between the values obtained colorimetrically and by the amino nitrogen method are calculated as imidazole propionic acid, the presence of 0.033 gm. (27.1 per cent), 0.01 gm. (8.1 per cent), and 0.016 gm. (13 per cent) of this substance is indicated for Flexner, Morgan, and Shiga respectively.

These organisms grew poorly, used glycerol only to a small extent, produced practically no acid, and removed very little ammonia from the solution. Histamine was not formed.

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[8] The (CR) standard is $\frac{10}{11}$ as intense as the (CR − MO) standard.

B. fæcalis alcaligenes I.—The color obtained with the histidine fraction was too red for histidine and the amino nitrogen value was 25 per cent lower than the colorimetric value. This suggests the presence of imidazole acetic, propionic, lactic, or acrylic acids. When the discrepancy between the values obtained colorimetrically and by the amino nitrogen method are calculated as imidazole propionic acid, the presence of 0.018 gm. (14.65 per cent) of this substance is indicated.

This organism grew so poorly that the medium was almost clear after 14 days of incubation. Some of the bacilli were, nevertheless, alive at the end of this period. Little or no use was made of the glycerol, and acid was not produced. Of the ammonia originally introduced, 90 per cent was recovered. Histamine was not formed.

B. fæcalis alcaligenes III.—This organism grew about as poorly as *B. fæcalis alcaligenes* I. During the first 3 days, 30 per cent of the histidine was removed. After that there was practically no change in the histidine content of the liquid. Imidazole propionic acid and histamine were not formed. Of the ammonia originally introduced, 90 per cent was recovered. The slight production of alkalinity during the last 11 days of incubation may have been caused by autolytic changes because apparently the active life of the organisms came to an end after 3 days of meager growth.

PART III.

On the Products Formed from Histidine by the Action of Organisms Other than Those Belonging to the Colon Typhoid Groups.

The organisms investigated were *Bacillus mucosus capsulatus* (2 strains), *Bacillus bifidus*, *Bacillus influenzæ*, *Bacillus proteus vulgaris* (2 strains), *Bacillus cloacæ*, *Streptococcus hæmolyticus*, (2 strains), Pneumococcus Types I, II, III, and IV, and *Bacillus tuberculosis* (5 strains). The behavior of these organisms on our synthetic medium is summarized in Table VIII.

B. mucosus capsulatus.—An excellent growth was obtained. This organism attacked the histidine immediately and so effectively that 75 per cent of it was destroyed in 14 days. The histidine seems to have been attacked because of its nitrogen. The following facts led us to this conclusion:

1. The ammonia concentration remained practically unchanged throughout the entire course of the experiment in spite of the fact that an unusually abundant growth was obtained. The nitrogen requirements of the organism must, therefore, have been obtained from the histidine.

2. Although the histidine was largely removed from the solution, none of its nitrogen appeared as ammonia and only a very small amount of it appeared in the amino condition. During the first few days, all of the nitrogen derived from the disrupted histidine was consumed. Toward the

TABLE VIII—*Miscel*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus mucosus capsulatus L. S., 3 days.	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	0.10 cc. = 7.3 mm. 0.20 " = 14.5 " Match perfect. 73%	1.33 cc. N_2 at 18° and 750 mm. 0.1535 gm. histidine dichloride. 76.8%
14 days.	0.10 cc. = 2.8 mm. 0.20 " = 5.6 " Match fair. 28%	0.20 cc. = 5.1 mm. 0.40 " = 10.2 " Match good. 25.5%	0.75 cc. N_2 at 25° and 745 mm. 0.083 gm. histidine dichloride. 41.5%
Bacillus mucosus capsulatus L. S. plus 0.10 gm. leucine, 14 days.	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match good. 80%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match good. 75%	1.46 cc. N_2 at 21° and 742 mm. 0.1655 gm. histidine dichloride. 82.7%
Bacillus mucosus capsulatus 27 (S), 3 days.	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. 75%	0.46 cc. N_2 at 23° and 752 mm. 0.052 gm. histidine dichloride. 26%
5 days.	0.20 cc. = 5.8 mm. 0.40 " = 11.6 " Match good. 29%	0.20 cc. = 4.9 mm. 0.40 " = 9.8 " Match good. 23%	0.80 cc. N_2 at 21° and 737 mm. 0.0894 gm. histidine dichloride. 44.7%
8 days.	0.50 cc. = 2.4 mm. Color brownish yellow. Match poor. 4.8%	0.50 cc. = 2.0 mm. 1.00 " = 4.0 " Match good. 4.0%	0.75 cc. N_2 at 21° and 737 mm. 0.0838 gm. histidine dichloride. 41.9%
Bacillus bifidus, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.7 mm. 0.20 " = 17.4 " Match perfect. 87%	1.55 cc. N_2 at 23° and 744 mm. 0.173 gm. histidine dichloride. 86.5%

*Colors matched against the (CR-MO) standard.

laneus Organisms.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	36.7 Hence the ≏ of 0.7 cc. of 0.1 N NH_3 was produced.	7.3	6.2
None.	None.	None.	36.7 An ammonia equilibrium was established after 7 days of growth.	7.3	6.8
None.	None.	None.	34 Hence the ≏ of 2 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.4
0.0365 gm. 29.7%	None.	None.	38.5 Hence the ≏ of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.2
None.	None.	None.	44 Hence the ≏ of 8 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.1
None.	None.	None.	49 Hence the ≏ of 13 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.0
None.	None.	None.	32.5 Hence the ≏ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus influenzæ, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.55 cc. N_2 at 32° and 746 mm. 0.165 gm. histidine dichloride. 82.5%
Bacillus proteus vulgaris 186, 7 days.	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match good. 76%	0.10 cc. = 6.9 mm. 0.20 " = 13.7 " Match good. 69%	1.13 cc. N_2 at 21° and 754 mm. 0.1294 gm. histidine dichloride. 64.7%
14 days.	0.20 cc. = 9.0 mm. 0.30 " = 13.7 " Color too yellow for histidine. 45%	0.20 cc. = 9.0 mm. 0.30 " = 13.5 " Match fair. Color slightly too yellow. 45%	0.78 cc. N_2 at 21° and 752 mm. 0.089 gm. histidine dichloride. 44.5%
Bacillus proteus vulgaris A. I. K., 14 days.	0.10 cc. = 8.1 mm. 0.20 " = 16.2 " Match perfect. 81%	0.10 cc. = 7.4 mm. 0.20 " = 14.7 " Match perfect. 74%	2.20 cc. N_2 at 20° and 738 mm. 0.2475 gm. histidine dichloride. 123.7%
Bacillus cloacæ I, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	1.85 cc. N_2 at 26° and 745 mm. 0.2036 gm. histidine dichloride. 101.8%
Streptococcus hæmolyticus 4A, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.5 mm. 0,20 " = 17.0 " Match perfect. 85%	1.51 cc. N_2 at 22° and 748 mm. 0.1705 gm. histidine dichloride. 85.2%
Streptococcus hæmolyticus II R, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.65 cc. N_2 at 28° and 746 mm. 0.180 gm. histidine dichloride. 90%

Continued.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
	None.	None.	31.5 Hence the ≏ of 4.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.3
None.	None.	None.	35 Hence the ≏ of 1 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
None.	None.	None.	40 Hence the ≏ of 4 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.1
None.	None.	None.	28 Hence the ≏ of 8 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.2
None.	None.	None.	30 Hence the ≏ of 6 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.5
None.	None.	None.	32.5 Hence the ≏ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.3
None.	None.	None.	33 Hence the ≏ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Pneumococcus Type I, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.48 cc. N_2 at 21° and 750 mm. 0.1684 gm. histidine dichloride. 84.2%
Pneumococcus Type I plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.54 cc. N_2 at 29° and 746 mm. 0.167 gm. histidine dichloride. 84%
Pneumococcus Type II, 14 days.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	0.10 cc. = 7.8 mm. 0.20 " = 15.5 " Match perfect. 78%	1.38 cc. N_2 at 32° and 748 mm. 0.1475 gm. histidine dichloride. 74%
Pneumococcus Type II plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. 89%	2.05 cc. N_2 at 32° and 746 mm. 0.2185 gm. histidine dichloride. 109.2%
Penumococcus Type III, 14 days.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. 79%	1.65 cc. N_2 at 32° and 748 mm. 0.1765 gm. histidine dichloride. 88.2%
Pneumococcus Type III plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.81 cc. N_2 at 32° and 748 mm. 0.1935 gm. histidine dichloride. 96.8%
Pneumococcus Type IV, 14 days.	0.10 cc. = 9.7 mm. 0.20 " = 19.3 " Match perfect. 97%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.47 cc. N_2 at 31° and 748 mm. 0.158 gm. histidine dichloride. 79%

Continued.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	33 Hence the ⇔ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
None.	None.	None.	33 Hence the ⇔ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	33 Hence the ⇔ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the ⇔ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the ⇔ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the ⇔ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
None.	None.	None.	33 Hence the ⇔ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Pneumococcus Type IV plus 0.10 gm. leucine, 14 days.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Match perfect. 97%	0.10 cc. = 7.9 mm. 0.20 " = 16.0 " Match perfect. 79%	1.60 cc. N_2 at 31° and 748 mm. 0.172 gm. histidine dichloride. 86%
Bacillus tuberculosis H. Sp., 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Color too red for histidine. 90%	1.23 cc. N_2 at 22° and 756 mm. 0.140 gm. histidine dichloride. 70%
Bacillus tuberculosis 1305, 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Color too red for histidine. 95%	0.95 cc. N_2 at 20° and 750 mm. 0.1085 gm. histidine dichloride. 54.2%
Bacillus tuberculosis 3161, 45 days.	0.10 cc. = 9.6 mm. 0.20 " = 19.3 " Color too red for histidine. 96%	0.10 cc. = 8.1 mm. 0.20 " = 16.2 " Color too red for histidine. 81%	0.95 cc. N_2 at 20° and 752 mm. 0.109 gm. histidine dichloride. 54.5%
Bacillus tuberculosis O. H., 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Color too red for histidine. 95%	1.09 cc. N_2 at 21° and 761 mm. 0.126 gm. histidine dichloride. 63%
Bacillus tuberculosis (Novy) K_1, 5 days.	0.10 cc. = 5.8 mm. 0.20 " = 11.5 " Match fair. Color too yellow. 58%	0.10 cc. = 4.7 mm. 0.20 " = 9.2 " Match fair. Color too yellow. 47%	1.27 cc. N_2 at 18° and 740 mm. 0.1446 gm. histidine dichloride. 72.3%
45 days.	1.00 cc. = 15.0 mm. Color brown. Match poor. 15%	1.00 cc. = 12.0 mm. Color brown. Match poor. 12%	0.81 cc. N_2 at 21° and 762 mm. 0.0936 gm. histidine dichloride. 46.8%

Concluded.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	32 Hence the ≎ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.015 gm. 12.2%	None.	None.	27 Hence the ≎ of 9 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.030 gm. 24.5%	None.	None.	28.5 Hence the ≎ of 7.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.020 gm. 16.3%	None.	None.	31 Hence the ≎ of 5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
0.023 gm. 18.7%	None.	None.	32.5 Hence the ≎ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	6.9
None.	None.	None.	38.5 Hence the ≎ of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.2
None.	None.	None.	37.5 Hence the ≎ of 1 cc. of 0.1 N NH_3 was removed during the 40 day period.	7.3	6.8

end of the 2 week period non-volatile amino nitrogen was liberated slightly faster than it was being consumed.

3. When leucine was added to the medium, along with the histidine, the growth of the organisms was augmented; but it was the leucine nitrogen that was removed from the solution, the histidine being largely unattacked. That the leucine nitrogen was removed is proved by the fact that the amino nitrogen value on the histidine fraction ran only slightly higher than the colorimetric value for histidine.

One might be led to assume that this organism cannot use ammonia to satisfy its nitrogen requirements. This is, however, not the case because a very good growth is obtained in a medium containing only glycerol and ammonium chloride together with the usual inorganic salts. In this case considerable ammonia is removed by the microorganisms. It seems that this organism used amino nitrogen in preference to ammonia nitrogen. Histamine and other imidazoles were not formed.

In addition to the above stock culture, two strains of *Bacillus mucosus capsulatus* were isolated in this laboratory, from the feces of two cases of colitis. They were quantitatively almost identical in their action. The horizontal Columns 4, 5, and 6 contain a summary of the behavior of one of them.

An excellent growth was obtained. The amino nitrogen value of the histidine fraction, obtained after 3 days of incubation, shows that a maximum of only 26 per cent of unchanged histidine could have been present. The color obtained with this fraction was too red to have been exclusively due to histidine, and the color value was far too high to agree with the amino nitrogen value; hence the presence of imidazole acetic, propionic, lactic, or acrylic acids is indicated. This discrepancy between the color value and the amino nitrogen value is equal to 0.0365 gm. (29.7 per cent) calculated as imidazole propionic acid. This organism therefore attacks the histidine in two distinct ways to obtain nitrogen. Some of the molecules are deaminized, others suffer a nuclear disruption. Some, but not much, ammonia is generated during the first 3 days.

During the next 2 days the imidazole propionic (?) acid formed at first was destroyed with the liberation of some non-volatile primary amine and considerable ammonia. The color obtained with the histidine fraction in this case was just like that obtained with histidine. We have assumed, therefore, that the color was at least *largely* due to histidine. If this conclusion is correct, the attack during this 2 day period was directed almost exclusively against the imidazole propionic acid formed during the first 3 days.

During the next 2 days the remaining histidine was destroyed almost completely with the liberation of ammonia. Histamine was not formed at any time.

The three strains of *Bacillus mucosus capsulatus* studied by us satisfied their nitrogen requirements at the expense of the amino-acid histidine in preference to ammonia. Two of the strains deaminized histidine and ruptured the nucleus simultaneously. The other strain merely ruptured the nucleus. Glycerol was, in every case, the chief source of carbon.

B. bifidus.—This organism grew very poorly on our medium, apparently because an available carbon source was lacking. The glycerol originally introduced seemed not to have been attacked. Of the histidine originally introduced, 87 per cent was recovered. There is no evidence that the nucleus was ruptured. The equivalent of 90 per cent of the initial ammonia was recovered. Histamine was not formed. When leucine was added to the medium the organisms grew for a few days during which time *all* the leucine nitrogen disappeared and some acid was produced. Histamine was not produced in this case.

B. influenzæ.—This organism grew very poorly on our medium. There is slight evidence for the formation of imidazole propionic (etc.) acids. Of the ammonia originally introduced, 91 per cent was recovered. Acid was not produced; glycerol was not utilized.

When leucine (0.10 gm.) was added to the medium, the organisms grew for a few days. They apparently found, in leucine, an easily available source of carbon and nitrogen because none of the leucine nitrogen could be accounted for at the end of 14 days. In this case 95 per cent of the histidine originally introduced was recovered. Histamine was not formed in any case.

B. proteus vulgaris 186.—This organism grew poorly on our medium apparently because an easily available source of carbon was lacking. Acid was not produced; glycerol was not attacked. The histidine was progressively destroyed, the excess nitrogen appearing as ammonia. The organisms must, therefore, have destroyed the histidine to obtain carbon. Histamine and other imidazoles were not formed.

B. proteus vulgaris A. I. K.—This organism grew very well on our medium. Glycerol was consumed and acids were produced. The great discrepancy between the color and amino nitrogen values for histidine is good evidence that a carboxylated amino compound was produced from the histidine. Since this organism was able to use ammonia as a source of nitrogen, the excessive amine production could hardly have been resorted to because of the nitrogen thus rendered available. The amines were more probably produced to lower the hydrogen ion concentration of the cell protoplasm. The equivalent of 77 per cent of the ammonia originally introduced was recovered. Histamine and other imidazoles were not formed.

B. cloacæ I.—As far as we could tell, this organism died shortly after it was introduced into our medium. The histidine and the glycerol were not attacked.

Streptococcus hæmolyticus 4A and II R.—Neither of these organisms grew perceptibly on our medium although they were still alive at the end of 14 days. Glycerol was not consumed; acid was not produced. About 85 per cent of the histidine originally introduced was recovered in each case. The initial ammonia concentration was reduced by only 10 per cent. Histamine and other imidazoles were not formed.

The results obtained with a strain of *Streptococcus viridans* were quantitatively almost identical with those obtained above.

Pneumococci, Types I, II, III, and IV.—These organisms grew poorly on our medium. They removed 15 to 20 per cent of the histidine originally introduced in such a way that the nitrogen cannot be accounted for either as NH_3 or as NH_2. There is no evidence that a nuclear rupture occurred. The constancy of the ammonia value indicates strongly that these organisms do not use it as a source of nitrogen. Histamine and other imidazoles were not formed.

When leucine (0.10 gm.) was added to the medium the organisms multiplied rapidly for a few days. The addition of leucine had no effect upon the NH_3 and pH values and the histidine was removed to about the same extent as in the leucine-free medium. The leucine, however, was almost completely destroyed in every case. This would seem to indicate that leucine is a good source of nitrogen and carbon for the pneumococcus while histidine, ammonia, and glycerol are poor sources of these elements.

B. tuberculosis H.Sp., 1305, 3161, and O.H.—These four strains of tubercle bacilli gave results that were qualitatively identical though quantitatively somewhat different. In every case there is good evidence that imidazole acetic, propionic, lactic, or acrylic acids were present. The color obtained with the histidine fraction was too red for histidine. The values obtained for histidine by the amino nitrogen method were always decidedly lower than those obtained colorimetrically. The discrepancies between the color values and the amino nitrogen values are equal to 0.015 gm. (12.2 per cent) for H.Sp., 0.030 gm. (24.5 per cent) for No. 1305, 0.02 gm. (16.3 per cent) for No. 3161, and 0.023 gm. (18.7 per cent) for O.H., calculated as imidazole propionic acid. The organisms grew very well on the surface of our medium. An excess of acid was not produced during this time interval although the glycerol had been largely destroyed. The ammonia consumption was distinctly different in the four cases, varying from 40 per cent in the case of H.Sp. to only 10 per cent in the case of O.H. Histamine was not produced in any case.

A striking contrast to these four organisms was that of a fifth strain which is widely known as K_1. This organism has been grown on synthetic media for so long—about 40 years—that it has lost its virulence entirely. It grows excellently on our medium, the growth not being confined to the surface. Chemically it did not behave like a typical tubercle bacillus as can be seen by examining the last two horizontal columns of Table VIII. This organism attacked the histidine immediately and so effectively that 53 per cent of it had been destroyed at the end of 5 days of incubation. Some of the histidine nitrogen was converted into ammonia; some appeared as a non-volatile, carboxylated amine. After 45 days of incubation only 12 per cent of the histidine originally introduced remained. An excess of acid was not formed although the glycerol had been destroyed. Imidazole propionic acid and histamine were not produced.

In concluding this part of the work we wish to call special attention to the fact that these experiments have all been carried out in an artificial synthetic medium. Our conditions are, at

best, highly artificial and the composition of our synthetic medium is far simpler than that of living tissue or even than that of the usual composite culture media. We are well aware that results obtained in such a medium must be applied carefully, if at all, to the general problem of amine production in the living organism. We feel, however, that our mode of procedure is justified by the fact that we have first to develop methods for estimating imidazoles and phenols under simple conditions before we can hope to estimate these substances in complex mixtures. The final answer to the question, "which microorganisms are probable amine producers in the human organism" can only be given after a medium has been used that contains all of the essential ingredients of a tissue or tissue extract. How definitely the amine production is influenced by the composition of the medium is shown clearly in the Part IV of this report where other amino-acids or peptones were added to our synthetic medium.

PART IV.

The Production of Histamine by Bacillus coli cystitis when Other Amino-Acids Are Present in the Medium Together with Histidine.

This investigation was undertaken to ascertain which amino-acids when added to our medium, augment the production of histamine and the growth of the microorganisms. The colon bacillus used in this work (*Bacillus coli cystitis*) was the strain employed by us in our original investigation.[1] We have found that this organism will always convert approximately 50 per cent of the histidine originally introduced into histamine when precautions are taken to have the initial pH of the medium 7.3 and to maintain a uniform temperature of 37°.

In the first experiment of this series we compared the carboxylase activity on our standard medium with that on a medium containing leucine and histidine in one case, and peptone and histidine in the other. The results obtained are summarized in Table IX.

The results obtained in the medium containing histidine as the only amino-acid call for very little special comment because they closely resemble those obtained and reported 2 years ago. In

TABLE IX—*Effect of Leucine and Peptone on the*

Composition of the medium.	Total color value of the test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Histidine dichloride, 0.2 gm. Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Match perfect. Color develops promptly. 112%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. 40%	0.85 cc. N_2 at 20° and 750 mm. 0.097 gm. histidine dichloride. 48.5%
Histidine dichloride, 0.2 gm. Leucine, 0.1 " Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. Color develops promptly. 103%	0.50 cc. = 4.5 mm. 1.00 " = 9.0 " Match poor. Color too yellow. 9.0%	0.72 cc. N_2 at 21° and 753 mm. 0.0822 gm. histidine dichloride. 41.1%
Histidine dichloride, 0.2 gm. Peptone (Witte), 0.05 " Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Match perfect. Color develops promptly. 112%	0.20 cc. = 3.0 mm. 0.40 " = 6.0 " Match good. 15%	0.65 cc. N_2 at 22° and 754 mm. 0.074 gm. histidine dichloride. 37%

* Colors matched against the (CR-MO) standard.

Produc ion of Histamine by Bacillus coli (cystitis).

Color value of the histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
			Before incubation.	After incubation.
		cc.	*pH*	*pH*
0.05 cc. = 13.8 mm. 0.10 " = 27.5 " Color developed like that of histamine. 0.092 gm. of histamine dichloride in entire test solution. 57% of histidine converted into histamine.	1.04 cc. N_2 at 20° and 754 mm. 0.0964 gm. histamine dichloride. 59.8%	27 Hence the ≎ of 9 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.4
Color too intense to run directly; hence 10 cc. were diluted to 100 cc. Of this solution 0.20 cc. = 7.6 mm. 0.40 " = 15.2 " Color develops like that of histamine. 0.1275 gm. of histamine dichloride in entire test solution. 79% of histidine converted into histamine.	2.55 cc. N_2 at 22° and 744 mm. 0.231 gm. histamine dichloride. 143%	25 Hence the ≎ of 11 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.5
Color too intense to run directly; hence 10 cc. were diluted to 100 cc. Of this solution 0.25 cc. = 9.3 mm. 0.50 " = 18.5 " Color develops like that of histamine. 0.125 gm. of histamine dichloride in entire test solution. 77.4% of histidine converted into histamine.	1.45 cc. N_2 at 23° and 755 mm. 0.133 gm. histamine dichloride. 82.4%		7.3	5.2

this case 57 per cent of the histidine originally introduced was converted into histamine. The agreement between the values obtained colorimetrically and by the amino nitrogen method is good.

Although the organisms multiplied rapidly in the above medium, they grew far better in a medium containing leucine or peptone together with the histidine. When leucine was present, 79 per cent of the histidine originally introduced was converted into histamine, as determined from the colorimetric reading an increase of 22 per cent over the leucine-free medium. The amino nitrogen value is obviously much too high to represent histamine alone. A portion of the leucine may have been converted into isoamylamine which would appear together with histamine in the amyl alcohol extract and thus raise the amino nitrogen value of the histamine fraction.

In the presence of peptone, 77 per cent of the histidine originally introduced was converted into histamine; hence leucine and peptone are about equivalent in their ability to promote histamine formation. In this case the check between the colorimetric and amino nitrogen values is sufficiently close to warrant the conclusion that other amines were not formed in appreciable quantities. In this case the acidity was also neutralized less perfectly than in the case of leucine where a large amount of amine production, other than histamine, was indicated.

In short then, we would conclude that the presence of either leucine or peptone stimulated the production of histamine. We can draw no conclusions from this experiment, as to the rôle played by the leucine or peptone in the amine production. We might conclude, with Jacoby,[9] that leucine was an easily available source from which carboxylase enzymes could be synthesized; but our experiment seems to indicate that the chief factor is the enormous increase in the speed of growth of the microorganisms which would, of course, increase the rate of acid production and hence render the early formation of histamine necessary to neutralize the acid.

We have grown *all* of the organisms discussed in the first three sections of this paper on a medium containing both leucine and histidine and have found that:

[9] Jacoby, M., *Biochem. Z.*, 1917, lxxxi, 332; lxxxiii, 74; lxxxiv, 358; 1918, lxxxvi, 329.

1. The addition of leucine always facilitates the growth of the organisms.

2. If the organisms produce no histamine in the absence of leucine, they will not produce histamine when leucine is present.

3. If the organisms produce histamine when leucine is absent, they will always produce 20 to 25 per cent more of this amine, in 2 weeks of incubation, when leucine is present. The leucine augments a power that already exists; it does not call forth a new enzymatic activity.

The second series was an enlargement upon the first for in this case all of the easily available amino-acids were employed; namely, glycine, alanine, cystine, leucine, arginine, glutamic acid, tyrosine, and tryptophane. In addition to the above amino-acids two flasks were prepared containing different makes of peptone; namely, Witte and Difco. The results obtained are summarized in Table X.

Table X shows that the histamine value on our standard medium was 20 per cent lower this time than it has been in any of our other experiments with this organism. The value on the leucine-histidine medium is also 20 per cent low. All of the flasks of *this* series were incubated at the same time in the same incubator. During the first few days the air in the incubator smelled strongly of hydrogen sulfide which was being evolved from the cystine-containing flask. We feel convinced that the retardation in speed of activity, and probably in rate of growth, was caused by the hydrogen sulfide. This conclusion is strengthened by the fact that only 2 per cent of histamine was formed in the cystine-histidine medium. The growth in this medium was also very meager. Since this hydrogen sulfide interference must have been approximately equivalent for all of the media, we believe that the values obtained are accurate in so far as they may be compared with one another. In discussing this series, a conversion of 31 per cent of the histidine originally introduced into histamine is considered to be the normal conversion on our standard medium. When more than 31 per cent of histamine was present, the amino-acid added can be considered to be an amine production *stimulator*, and *vice versa*. The amino nitrogen values on both the histidine and histamine fractions have, in every case, been calculated as histidine and as histamine, respectively, in spite of the fact

TABLE X—*Effect of Amino-Acids and Peptones on*

Composition of the medium.	Histidine dichloride, 0.2 gm. KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Glycine, 0.065 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Alanine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Leucine, 0.115 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Cystine, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
Total color value calculated as % of histidine dichloride. (0.20 gm. = 100%), *per cent.*	112	108	100	112	96
Unchanged histidine colorimetric method. (0.20 gm. = 100%), *per cent.*	60	43	30	37	87
Amino nitrogen value of histidine fraction calculated as % of histidine dichloride. (0.20 gm. = 100%), *per cent.*	74	102.5	154	55	147
Histidine converted into histamine (colorimetric method) *per cent.*	31	37.2	51	49.6	2.06
Amino nitrogen value of histamine fraction calculated as % of histamine dichloride, *per cent.*	32	49	70.7	152	

the Production of Histamine by Bacillus coli (cystitis.)

Histidine dichloride, 0.2 gm. Glutamic acid HCl, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Arginine carbonate, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tyrosine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tryptophane, 0.18 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Witte), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Difco), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
105	110		99	103	97
62	52	11.5	60	40	50
115.5	109.8	10.6	79.5	60.6	67.1
21.7	41	31.4	26.3	42	42
42.5	108.4			56.6	62.8

Composition of the medium.	Histidine dichloride, 0.2 gm. KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Glycine, 0.065 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Alanine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Leucine, 0.115 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Cystine, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
0.10 N HCl neutralized by NH_3 from the entire test solution, *cc.*	20.5 Hence the ≈ of 16 cc. of 0.1 N NH_3 was used by microorganisms.	16.5 Hence the ≈ of 19.5 cc. of 0.1 N NH_3 was used by microorganisms.	17 Hence the ≈ of 19 cc. of 0.1 N NH_3 was used by microorganisms.	23.7 Hence the ≈ of 12.3 cc. of 0.1 N NH_3 was used by microorganisms.	9.2 Hence the ≈ of 26.8 cc. of 0.1 N NH_3 was used by microorganisms.
pH after 14 days of incubation. The initial pH was 7.3 in all cases, *pH.*	5.5	5.5	5.6	5.9	5.3
Tyrosine converted into volatile phenols (colorimetric method), *per cent.*					
Tyrosine converted into aromatic hydroxy-acids. Calculated as oxyphenyllactic acid (colorimetric method) *per cent.*					
Unchanged tyrosine (colorimetric method), *per cent.*					
Tyrosine converted into tyramine (colorimetric method) *per cent.*					

Concluded.

Histidine dichloride, 0.2 gm. Glutamic acid HCl, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Arginine carbonate, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tyrosine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tryptophane, 0.18 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Witte), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Difco), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
17.5 Hence the ∞ of 18.5 cc. of 0.1 N NH_3 was used by microorganisms.	19.5 Hence the ∞ of 16.5 cc. of 0.1 N NH_3 was used by microorganisms.	23.5 Hence the ∞ of 12.5 cc. of 0.1 N NH_3 was used by microorganisms.	20 Hence the ∞ of 16 cc. of 0.1 N NH_3 was used by microorganisms.	24.5 Hence the ∞ of 11.5 cc. 0.1 N NH_3 was used by microorganisms.	22.2 Hence the ∞ of 13.8 cc. of 0.1 N NH_3 was used by microorganisms.
5.9	5.6	5.7	5.4	5.5	5.4
		None.			
		3.4			
		72.6			
		None.			

that the values must obviously be high because of the presence of the other amino-acid or peptone. These amino nitrogen figures are of value because they give some information about the fate of the added amino-acid.

Glycine and Histidine.—The organisms grew distinctly better in a medium containing glycine and histidine than they did in a medium containing only histidine. The histamine production was increased by 6.2 per cent. Most of the glycine nitrogen remained in the histidine fraction in the primary amino condition which indicates that only a small amount of this amino-acid was utilized by the microorganisms.

Arginine and Histidine.—The microorganisms grew very much better in this medium than they did in the one containing glycine and histidine. The histamine production was increased by 10 per cent. The amino nitrogen figures indicate that over half of the arginine was converted into some product that passed into amyl alcohol from a strongly alkaline solution. The arginine may, therefore, also have been decarboxylated.

Peptone and Histidine (Witte and Difco).—The presence of either of the above peptones is a great stimulus to the rate of growth of these bacilli, the growth being about equivalent to that obtained in the arginine-histidine medium. The histamine production was increased by 11 per cent in each case.

Alanine and Histidine.—The organisms grew even better in a medium containing alanine than they did in one containing peptone. In this case the histamine production was increased by 20 per cent, the highest value obtained with any of the amino-acids. The alanine used in this work was a mixture of equal parts of the *d*- and *l*-varieties. Two equivalents of this alanine were used. It is interesting to note that one and a quarter equivalents remained, probably as alanine, in the histidine fraction and that a maximum of one-fifth of an equivalent passed into amyl alcohol, possibly as an amine. In short, then, over half of the introduced available nitrogen was consumed by the microorganisms. (We have assumed that *l*-alanine did not serve as a food.)

Leucine and Histidine.—The growth obtained in this case was slightly better than that obtained on the histidine-alanine medium. The histamine production was increased by 18.6 per cent which is almost identical with that obtained on the alanine-histidine

medium. Most of the leucine nitrogen appeared in the histamine fraction which indicates that the leucine was also decarboxylated.

Tryptophane and Histidine.—The growth in this medium was about as dense as that obtained in the peptone-histidine medium; but the histamine production was *decreased*, not increased. The 26.3 per cent of histamine obtained in this case is about 5 per cent less than that obtained in a medium containing histidine as the only amino-acid. We anticipated some trouble with our colorimetric process in this case; but we were relieved to find that tryptophane, or its decomposition products, did not interfere in any way with the color production.

Glutamic Acid and Histidine.—The growth on this medium was slightly less than that on the tryptophane-histidine medium. The presence of glutamic acid *decreases* the histamine production by about 9 per cent. The amino nitrogen figures indicate that most of the glutamic acid nitrogen was still attached to a carboxylated molecule.

Cystine and Histidine.—The growth on this medium was very meager. The presence of cystine decreases the histamine production by 29 per cent so that the formation of this amine is almost nil.

Tyrosine and Histidine.—The fact that tyrosine and its derivatives give a color with alkaline *p*-phenyldiazonium sulfonate, made it necessary for us to modify our usual procedure in this case. The method employed was briefly as follows:

1. The filtered medium, after the usual treatment with 1 cc. of 95 per cent sulfuric acid, was subjected to a distillation, under ordinary pressure, the distillate being collected and examined colorimetrically[10] for volatile phenols. Phenols were absent.

2. The contents of the distilling flask were transferred to a glass dish and evaporated on the water bath. The residue was transferred, with water, to a 25 cc. precision cylinder and diluted to 25 cc. This is the *test liquid.*

3. Of this acid *test liquid* 10 cc. were transferred to a 35 cc. extraction bottle and extracted ten times with ether, using 20 cc. for each extraction. The combined ether extracts were treated

[10] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 235.

with 25 cc. of water, the ether being then removed by distillation at first under ordinary pressures and then *in vacuo*. The solution so obtained was transferred, with water, to a 100 cc. precision cylinder and diluted to 100 cc. It was tested, colorimetrically,[10] for aromatic hydroxy-acids. In this way 3.4 per cent of the tyrosine originally introduced was found to have been converted into oxyphenyllactic acid.

4. The aqueous liquid obtained above, that had been freed from aromatic hydroxy-acids by extraction with ether, was transferred to a 250 cc. Pyrex flask with 90 cc. of water. Silver nitrate, 10 cc. of a 20 per cent solution, was added and the resulting mixture treated with 12 gm. of barium hydroxide in 50 cc. of warm water. The dark brown mixture was filtered, the precipitate being washed with a cold saturated solution of baryta.[11]

This divides the material into two fractions; *the silver precipitate*, which will contain all of the histamine and which should contain most of the histidine, and *the silver filtrate* which should contain all of the tyrosine and tyramine.

5. *The silver precipitate* was suspended in water and treated with an excess of 37 per cent HCl and Na_2SO_4 as has been previously described.[11] The resulting mixture was filtered after 2 hours of digestion on the water bath and the filtrate evaporated on the water bath. The residue was then transferred to an extraction bottle with 10 cc. of water and treated just like any of the histidine test liquids. It was found to contain 11.5 per cent of histidine and 31.4 per cent of histamine. The recovery of imidazoles was only 43 per cent. We have not checked up on the silver precipitation method for histidine sufficiently to be certain that some histidine may not have remained unprecipitated. We are certain that the histamine figure is accurate because the method employed in this experiment was identical with that used in the quantitative precipitation of histamine in some of our earlier work.[11]

6. *The silver filtrate*. The barium and silver ions were exactly removed with H_2SO_4 and HCl, respectively. The filtrate from $BaSO_4$ and AgCl was neutralized with sodium hydroxide and evaporated on the water bath. The residue was transferred to

[11] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543.

an extraction bottle with 10 cc. of water. The subsequent separation of tyramine from tyrosine and the colorimetric estimation of each substance in its respective fraction was then carried out as described in the following paper.[12] Tyramine was found to be absent. Of the tyrosine originally introduced, 72.6 per cent was recovered.

The histamine production on this medium proceeded at a rate identical with that on a tyrosine-free medium. *Tyrosine neither augmented nor retarded the histamine formation. Tyrosine was not decarboxylated.*

It appears then, that the decarboxylation of histidine is influenced by the presence of other amino-acids in all of the three possible ways. *Tyrosine is without effect. Leucine, alanine, arginine, and glycine increase the rate of decarboxylation. Of these, leucine and alanine are by far the most efficient. Cystine, glutamic acid, and tryptophane decrease the rate of decarboxylation. Of these, cystine is by far the most efficient.* The rate of decarboxylation is not entirely coincident with the rate of growth of the microorganisms because, with the exception of cystine, all of the amino-acids augmented the growth of the organisms; but tryptophane and glutamic acid *decreased* the histamine production.

SUMMARY.

1. The behavior of a large number of microorganisms has been studied on a liquid medium consisting of histidine dichloride (0.2 gm.), ammonium chloride (0.2 gm.), potassium nitrate (0.1 gm.), potassium dihydrogen phosphate (0.4 gm.), sodium chloride (0.8 gm.), sodium sulfate (0.02 gm.), sodium bicarbonate (0.4 gm.), calcium chloride (0.01 gm.), and glycerol (4.0 cc.), in a total aqueous volume of 200 cc.

2. The organisms studied were *Bacillus coli communior* (7 strains), *Bacillus coli communis* (5 strains), *Bacillus lactis aerogenes* (5 strains), *Bacillus acidi lactici* (12 strains), *Bacillus enteritidis*, *Bacillus typhosus*, *Bacillus paratyphosus* A (3 strains), *Bacillus dysenteriæ* Flexner, Morgan, and Shiga, *Bacillus fæcalis alcaligenes* I and III, *Bacillus mucosus capsulatus* (3 strains), *Bacillus bifidus*, *Bacillus influenzæ*, *Bacillus proteus vulgaris* (2 strains), *Bacillus cloacæ*, *Streptococcus hæmolyticus* and *viridans*, pneumococci (Types I, II, III, and IV), and *Bacillus tuberculosis* (5 strains).

[12] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 193.

3. *Of the twenty-nine strains of coli*—in the narrower sense—that we studied, *six* were able to convert histidine into histamine on our synthetic medium. None of the other organisms were histamine producers.

4. Five members of the colon group—in the narrower sense—gave quantitative evidence that an alkali-stable, carboxylated triamino compound was formed from the histidine. This compound may have the formula

$$\begin{array}{l} HC - NH_2 \\ \;\;\| \\ HC - NH_2 \\ \;\;| \\ \;\;CH_2 \\ \;\;| \\ HCNH_2 \\ \;\;| \\ \;\;COOH \end{array}$$

None of the other organisms gave quantitative evidence for the formation of such a compound; but most of them gave results that would lead one to suppose that such a compound was formed to some extent as an intermediate in the decomposition of histidine.

5. Imidazole acetic, propionic, lactic, or acrylic acid was formed by *Bacillus paratyphosus* A (1 strain), *Bacillus dysenteriæ* Flexner, Morgan, and Shiga, *Bacillus fæcalis alcaligenes* I, *Bacillus mucosus capsulatus* (2 strains), and *Bacillus tuberculosis* (4 strains). We cannot say for certain which of the above acids was produced in any case because our method does not differentiate between them.

6. The addition of leucine to the standard medium facilitates the growth of all of the organisms studied.

7. If the organisms produce no histamine when leucine is absent, they do not produce histamine when leucine is present.

8. If the organisms produce histamine when leucine is absent, they produce 20 to 25 per cent more of this amine, in 2 weeks of incubation, when leucine is present. The leucine augments a power that already exists; it does not create a new enzymatic activity.

9. The addition of alanine, leucine, arginine, glycine, or peptone—either Witte or Difco—to the standard medium, augments the growth of the colon bacillus and increases the yield of histamine.

When glutamic acid or tryptophane are added to the standard medium, the growth of the organisms is augmented; but the output of histamine is decreased. Cystine is unfavorable to the growth of the colon bacillus. The presence of this amino-acid reduces the yield of histamine to almost nil. The remarkable retarding influence of cystine on the growth of the microorganisms and on the histamine production by the colon bacillus seems to be due to the hydrogen sulfide which is evolved in quantity during the first few days of incubation. The addition of tyrosine to our standard medium seems to have no influence upon the rate of histamine production.

We wish to acknowledge the technical assistance of Miss Edith H. Bell throughout this work.

STUDIES ON PROTEINOGENOUS AMINES.

XIII. ON THE ELECTRONIC INTERPRETATION OF CERTAIN BIOCHEMICAL PHENOMENA.

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In our work on biological problems we have frequently encountered reactions that could not have been readily explained on the basis of the accepted structural formulas of the compounds involved, because these formulas are incomplete indices of the degree of oxidation of the carbon atoms. We were led, therefore, to a consideration of the interatomic forces and we have found that the electronic formulas so derived have been of great service in clarifying certain puzzling phenomena.

Parts I and II of this paper contain an electronic explanation of the carboxylase activity of yeast and of the fact that the fatty acids oxidize predominantly in the beta position in the animal body. In Part III we will attempt to show how the electronic formulas for some of the biologically important compounds can be derived.

PART I.

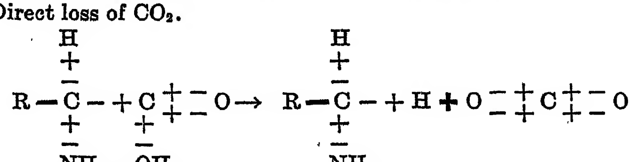
On the Carboxylase Activity of Microorganisms with Particular Reference to Yeasts.

One of the main sources of the formation of amines by the living cell is the decarboxylation of amino-acids. While chiefly accomplished through the activity of microorganisms, there is evidence that this carboxylase activity is also part of the life process of some higher plants and of certain animals. The occurrence of *p*-oxyphenylethylamine in several species of mistletoe[1] (*Phoradendron flavescens*, *Phoradendron villosum*, and *Phoradendron californicum*) and the presence of the same amine in the salivary gland and secretion of some Cephalopoda[2] may be recalled in this connection as evidence for this statement. Up to the present time, however, enzymes capable of decarboxylating amino-acids to amines *in vitro* have not been isolated from either unicellular or multicellular organisms. While the isolation of such an enzyme of the carboxylase type would be of interest, it is doubtful if this accomplishment would clarify the mechanism of decarboxylation any more than the discovery of zymase has enhanced the problem of alcoholic fermentation and of the breakdown of sugars. To obtain a better understanding of the enzymatic activities of the living cell it would seem far more promising to undertake a systematic study of the intermediates formed in the catabolism of higher molecular complexes, especially if we could understand the interatomic forces which govern this breakdown.

The decarboxylation of amino-acids, with the formation of carbon dioxide and the corresponding amine might, theoretically, be accomplished in any of the following ways.

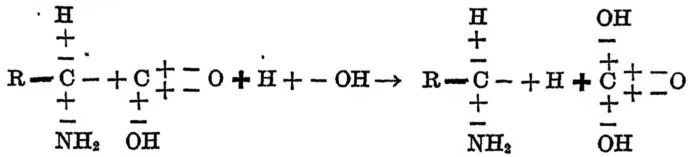
1. With a quadruply positive carboxyl group.

A. Direct loss of CO_2.

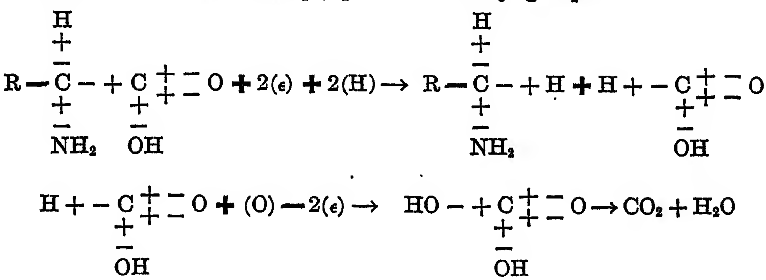


[1] Crawford, A. C., and Watanabe, W. K., *J. Biol. Chem.*, 1916, xxiv, 169.
[2] Henze, M., *Z. physiol. Chem.*, 1913, lxxxvii, 51.

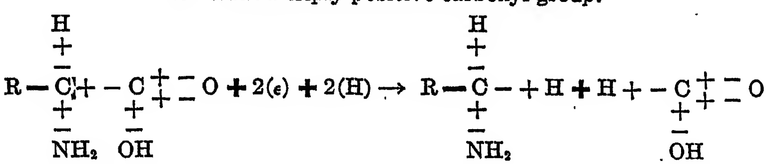
B. Hydrolysis.



C. Reduction of the quadruply positive carboxyl group.

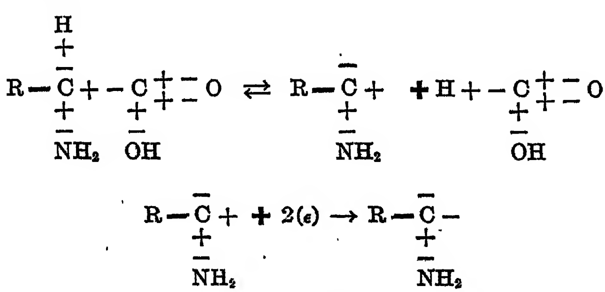


2. With a triply positive carboxyl group.



The formic acid would then give CO_2 and H_2O, by oxidation, as in C above.

The above equation tells nothing about the mechanism of the reaction. Following Nef, we might consider the above reaction to proceed as follows.[3]



[3] Koessler, K. K., *Proc. Inst. Med. Chicago*, 1920, iii, 46.

$$\begin{array}{ccccc} & & & & \mathrm{H} \\ & & & & + \\ & - & & & - \\ \mathrm{R}- & \mathrm{C} & - + 2(\mathrm{H}+) \rightarrow & \mathrm{R}- & \mathrm{C} - + \mathrm{H} \\ & + & & & + \\ & - & & & - \\ & \mathrm{NH_2} & & & \mathrm{NH_2} \end{array}$$

For reasons that will be discussed later, the schematic possibility represented by equation (1, C) is hardly worthy of consideration because it involves the reduction of a quadruply positive carbon atom. As far as we now know, only the chlorophyll-containing plants have this power.

A consideration of Types I and II shows that the kind of reaction resorted to must depend upon the direction of the electrical field between the carboxyl group and its neighboring carbon atom. If we were certain that the carboxyl group in all amino-acids was negative with respect to its neighboring carbon atom as in formula (2), we could state with a fair degree of assurance that the decarboxylation of amino-acids must be associated with an oxidation reduction process. Unfortunately, we are at present unable to make a definite statement as to the electrical conditions that prevail in any amino-acid (see under allyl chloride, pages 231 to 233). It seems indeed, as if the charge on the carboxyl group is not the same for all amino-acids.

Although we are, therefore, unable at present to give an electronic interpretation of the carboxylase activity as applied to amino-acids, the electronic point of view offers a fascinating explanation for a related type of carboxylase activity.

Neuberg[4] and his coworkers have shown that yeasts and yeast extracts have the faculty of decarboxylating pyruvic and many other organic acids with the formation of carbon dioxide and aldehydes; *e.g.*, $CH_3-CO-COOH \rightarrow CO_2 + CH_3-CHO$.

Karczag and Breuer[5] have shown that many bacteria, although they ferment pyruvic acid with gas formation, produce no aldehyde. The gas produced, instead of being pure CO_2 as in the case of the yeasts, consists of hydrogen to the extent of 90 per

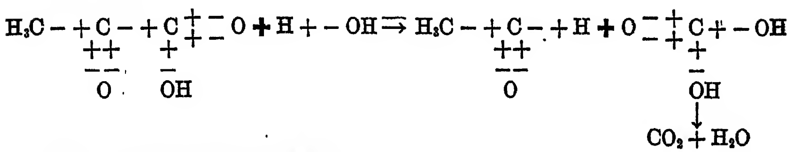
[4] Neuberg, C., and Hildesheimer, A., *Biochem. Z.*, 1911, xxxi, 170. Neuberg, C., and Tir, L., *Biochem. Z.*, 1911, xxxii, 323. Neuberg, C., and Karczag, L., *Biochem. Z.*, 1911, xxxvi, 68, 76; xxxvii, 170. Neuberg, C., and Kerb, J., *Biochem. Z.*, 1912, xlvii, 413, 405.

[5] Karczag, L., and Breuer, E., *Biochem. Z.*, 1915, lxx, 320.

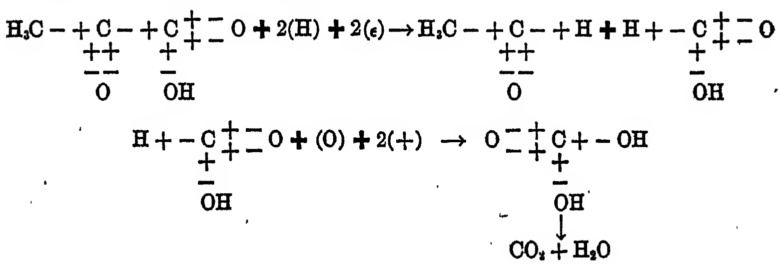
cent. Obviously, then, the action of yeasts on pyruvic acid is radically different from that of the investigated bacteria. The authors believe that some light is shed upon these facts and others to be presented later, by a consideration of the electronic formulas of these substances.

Acetaldehyde and carbon dioxide could be obtained from pyruvic acid by either of the three following processes.[6]

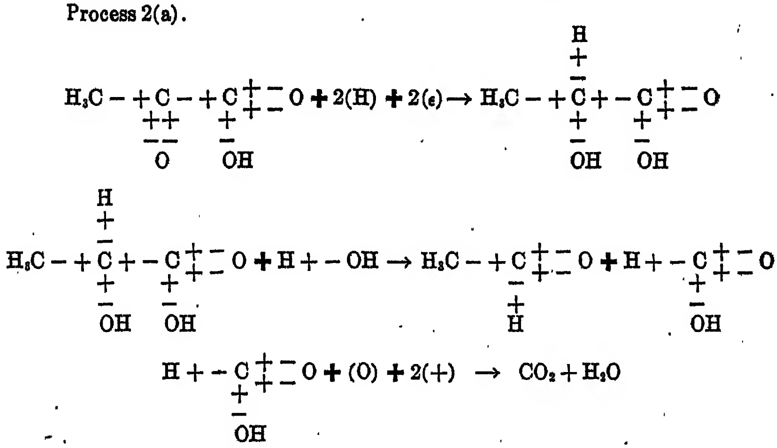
1. Hydrolysis.



2. Reduction followed by oxidation.



Process 2(a).



[6] The proof of the electronic formulas for the compounds used in the equations throughout this paper is given in Part III.

The decomposition of pyruvic acid by yeast *cannot* occur according to process (2) or (2, a) for the following reasons.

Process (2) demands the intermediate formation of formic acid *by the reduction of a quadruply positive carbon atom.* Such a reduction would be equivalent to the direct conversion of CO_2 to formic acid. Up to the present time only the chlorophyll-containing plants have been proved to be capable of reducing CO_2, and they can do so only in the presence of sunlight. This process is, therefore, highly improbable from the start. If we pass lightly over this first serious objection, we come to another that is just as serious. The formulation demands that the formic acid formed at first must then be oxidized to CO_2. That bacteria have this power of oxidation is clearly proved by the work of Omelianski[7] and Pakes and Jollyman.[8] In every case, however, hydrogen is evolved along with the CO_2. Hydrogen is never evolved in the early stages of yeast fermentations. Although the evolution of CO_2 from pyruvic acid by yeast is extremely rapid so that the fermentation is practically over in 24 hours irrespective of the variety of yeast employed, Neuberg and Tir[4] have shown that certain of these yeasts will evolve no gas at all from sodium formate solutions and that none of them evolves more than traces of CO_2 in the course of 24 hours.

An objection might be raised to these conclusions from the work of Neuberg and Tir because these authors used a pure solution of sodium formate in which no real fermentation could occur. It is conceivable that the oxidation of formic acid might take place only in the presence of sugar. Franzen and Steppuhn[9] have shown conclusively that when sodium formate is mixed with a nutrient medium rich in sugar, most of the pure cultures of yeast employed, although they fermented the sugar vigorously, either did not ferment the added formic acid at all or did so only to a very small extent. In no case was the destruction of the formic acid *rapid* enough to conclude that this substance was an intermediate product in the fermentation of sugar. Formulation (2) is, therefore, highly improbable.

[7] Omelianski, W., *Centr. Bakt., 2te Abt.*, 1904, xi, 177, 256, 317.

[8] Pakes, W. C. C., and Jollyman, W. H., *J. Chem. Soc.*, 1901, lxxix, 322, 386, 459.

[9] Franzen, H., and Steppuhn, O., *Z. physiol. Chem.*, 1912, lxxvii, 129.

Process (2, a) demands the intermediate formation of lactic acid. That the reduction of pyruvic acid to lactic acid might be resorted to by *bacteria* in case the pyruvic acid was being used by them as a source of carbon is very possible because as can be seen from the formula, this is an indirect but simple method for converting an *unavailable quadruply positive carboxyl group into one that is triply positive.* That yeasts do *not* resort to this process, in the case of pyruvic acid, is proved by the fact that *lactic acid is not fermented* at all by some yeasts and only to a very slight extent by most (see article by Neuberg and Tir[4] and Buchner and Meisenheimer[10]).

Even if lactic acid were fermented by yeast, the formic acid difficulty would still be insurmountable (see above under process (2)). Apparently then, formula (2, a) cannot represent what happens when pyruvic acid is fermented by yeast.

Process (1) is the only one left and its very simplicity is exactly what one would expect from a reaction that proceeds so smoothly and rapidly. The above considerations have convinced the authors that *the fermentation of pyruvic acid by yeast is accomplished by a purely hydrolytic process.*

From the ordinary structural formula for lactic acid it is difficult to see why a similar hydrolysis does not occur in this case with the formation of CO_2 and alcohol. The hydrolysis of lactic acid with *dilute sulfuric* acid, which takes place quite as readily as the similar hydrolysis of pyruvic acid, gives acetaldehyde and formic acid. The electronic formulas for these two compounds show clearly why CO_2 could not be obtained by the hydrolysis of lactic acid and they *show moreover why yeast cannot hydrolyze lactic acid at all.* Lactic acid contains a *triply* positive carboxyl group while pyruvic acid contains one that is *quadruply* positive. Apparently then, *yeast can hydrolyze only those acids containing quadruply positive carboxyl groups.*

With this idea in mind it is easy to see why oxalacetic acid should give acetaldehyde and CO_2, acetone dicarbonic acid should give acetone and CO_2, and in general why all of the α-ketonic acids should give an aldehyde and CO_2 as primary products because *they all contain quadruply positive carboxyl groups.* A striking confirmation of this statement is found in the fact that *glyoxylic*

[10] Buchner, E., and Meisenheimer, J., *Ber. chem. Ges.*, 1910, xliii, 1773.

acid, the mother substance of the α-ketonic acids, *is practically not fermented by yeast.*[4] *This acid has been shown to contain a negative carboxyl group and it could not give CO_2 on hydrolysis.*

No attempt has been made to explain the mechanism of hydrolysis. Just why pyruvic acid should hydrolyze readily whereas acetic acid must be fused with an alkali to hydrolyze it is not explained by the electronic formulas. The failure of yeast to ferment an acid does not prove that the acid contains a *negative* carboxyl group. On the other hand, if yeast does split CO_2 from an acid *readily* it is fairly safe to conclude that the acid contains a quadruply positive carboxyl group. Acetic acid is fermented very slowly by yeast. Neuberg and Tir make the statement that the gas evolved is only partially absorbed by sodium hydroxide. It would be of interest to ascertain if the unabsorbed gas was methane because if it was methane, the yeast would have proved itself capable of hydrolyzing even this very resistant acid.

PART II.

On the Beta Oxidation of Fatty Acids in the Animal Body.

The work of Knoop, Dakin, and others[11] leaves little doubt that fatty acids undergo oxidation predominantly in the beta position in the animal body. Just why the living cells and hydrogen peroxide should oxidize the beta carbon atom by preference while the halogens react only with the alpha hydrogen atoms of the fatty acids is a question that has been much discussed in the past. It occurred to the authors that this tendency toward beta oxidation in the body (and by hydrogen peroxide) might be closely associated with the electrical structure of the molecule.

The carbon atom can occur in five stages of oxidation; namely,

$$1.\ -\overset{-}{\underset{-}{C}}- \qquad 2.\ -\overset{-}{\underset{-}{C}}+ \qquad 3.\ -\overset{-}{\underset{+}{C}}+ \qquad 4.\ -\overset{+}{\underset{+}{C}}+ \qquad 5.\ +\overset{+}{\underset{+}{C}}+$$

Carbon dioxide is the simplest substance that contains carbon in the completely oxidized condition represented by form (5). Neither microorganisms nor the cells of the animal organism seem to have the faculty of reducing CO_2. Only the chlorophyll-

[11] The references to the original literature are given in Dakin's monograph (Dakin, H. D., Oxidation and reductions in the animal body, London, 1912).

containing plants have been proved to be capable of reducing CO_2 to formaldehyde and they can do so only in the presence of sunlight.[12] Forms (2), (3), and (4) seem to be oxidizable or reducible by living matter depending upon the conditions of the experiment. At present it seems advisable to make no definite statement as to which of these forms is most readily oxidizable.

The work of numerous investigators seems to indicate that form (1) is not *readily* oxidized by living matter. Thus Karczag and Breuer[5] showed that oxalacetic, acetone dicarbonic, and acetic acids were not changed by a large number of microorganisms. Of these acids, acetone dicarbonic and acetic acids contain only quadruply negative and quadruply positive carbon. Oxalacetic acid has one quadruply negative, two quadruply positive, and one triply positive carbon atom. From this it would appear that quadruply negative carbon was not readily oxidized by bacteria.

Although acetic, acetoacetic, and malonic acids, all of which contain only quadruply negative and positive carbon, are completely oxidized in the normal animal body, acetone is attacked with difficulty[13] and phenylacetic acid is not oxidized at all.[14] Acetone has been proved to contain only quadruply positive and quadruply negative carbon and phenylacetic acid must have the formula

$$\begin{array}{ccccc}
 & H & & & \\
 & + & & & \\
 & - & & & \\
C_6H_5 + - & C & - + & C & \begin{smallmatrix}+ & -\\ + & -\end{smallmatrix}\ O \\
 & - & & + & \\
 & + & & - & \\
 & H & & OH &
\end{array}$$

It seems therefore, that even the *normal* body cells frequently find it *difficult* to oxidize quadruply negative carbon. From these facts two conclusions can be drawn.

1. If a compound containing quadruply negative carbon together with other more easily oxidizable varieties were fed to a *normal* animal one would expect a predominant and initial oxidation of the more easily oxidizable carbon atoms. In the case

[12] For a critical review of this subject see Bayliss, W. M., Principles of general physiology, London, New York, Bombay, Calcutta, and Madras, 1915, 564.

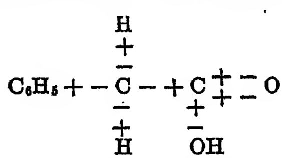
[13] Geelmuyden, H. C., *Z. physiol. Chem.*, 1897, xxiii, 431.

[14] Knoop, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 150.

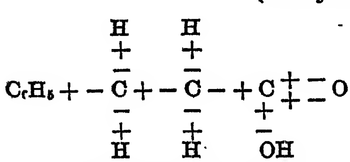
of *most* compounds, these primary oxidation products would then undergo further oxidation, because of the vigorous oxidizing conditions that prevail in the normal organism, so that an excretion of more than traces of these partially oxidized products is hardly to be expected.

2. In certain diseases, notably diabetes, the oxidizing power of the body cells is greatly diminished. Under these conditions the difficultly oxidizable quadruply negative carbon atoms might escape oxidation, at least partially, while the more easily oxidizable carbon atoms in the same molecule might be oxidized. These partially oxidized products, with the quadruply negative carbon still intact, should then be excreted. It is a well known fact that acetone and acetoacetic acid are excreted in large quantities by diabetics. One would expect also that acetic acid, acetone dicarbonic acid, citric acid, and a large number of other substances containing quadruply negative carbon should be at least partially excreted unchanged by diabetics. Citric acid contains one triply positive carbon atom. It might, therefore, be eliminated as acetone dicarbonic acid or if the carboxylase activity was too great it should surely appear as acetone. (The carboxylase of yeast readily decomposes acetone dicarbonic acid into carbon dioxide and acetone by a purely hydrolytic process as has been shown in Part I. A similar decomposition might occur in diabetics.)

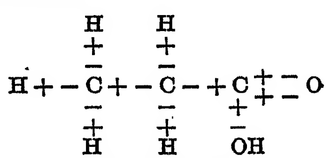
With the idea in mind that quadruply negative carbon is not readily oxidized by the body cells, we can now consider the probable primary oxidation products of a few acids whose electronic formulas shall be repeated here for the sake of simplicity.



(Phenylacetic acid.)



(Phenylpropionic acid.)



(Propionic acid.)

$$\begin{array}{lcccc}
 & H & H & H & \\
 & + & + & + & \\
 & - & - & - & \\
C_6H_5 + & -C- & +C+ & -C- & +C\begin{smallmatrix}+&-\\+&-\end{smallmatrix}O \\
 & - & - & - & + \\
 & + & + & + & - \\
 & H & H & H & OH
\end{array}
\qquad
\begin{array}{lcccc}
 & H & H & H & \\
 & + & + & + & \\
 & - & - & - & \\
H + & -C- & +C+ & -C- & +C\begin{smallmatrix}+&-\\+&-\end{smallmatrix}O \\
 & - & - & - & + \\
 & + & + & + & - \\
 & H & H & H & OH
\end{array}$$

(Phenylbutyric acid.) (Butyric acid.)

Phenylacetic acid contains the perfectly stable quadruply positive carboxyl group, the very resistant benzene ring, and the presumably difficultly oxidizable quadruply negative alpha carbon atom. This compound should, therefore, not be readily oxidized even by the normal body cells. As a matter of fact phenylacetic acid is *not* oxidized by the normal body cells.

Phenylpropionic acid is *partially oxidized only in the beta position.* This triply negative carbon atom should be the most vulnerable position in the molecule. An oxidation at this point would lead finally to benzoylacetic acid which by one type of hydrolysis would give benzoic acid and acetic acid. Knoop found that phenylpropionic acid was converted into benzoic acid in the normal animal while Dakin was also able to isolate a small amount of benzoylacetic acid under similar conditions.

Phenylbutyric acid *is partially oxidized only in the beta position.* This doubly positive carbon atom should be the most easily attacked position in the molecule. An oxidation at this point would lead to the formation of phenylacetoacetic acid which by one type of hydrolysis would give phenylacetic acid and acetic acid. Knoop found that phenylacetic acid was formed from phenylbutyric acid in the normal animal.

In propionic acid *it is again the beta position that is partially oxidized* and therefore, should be most readily still further oxidized. The final product of the oxidation would be malonic acid. Malonic acid is, however, very readily oxidized by the normal body cells; hence the isolation of a definite end-product is hardly to be expected in the normal animal. As a matter of fact propionic acid is completely oxidized in the normal animal without leaving a clue as to the mode of its decomposition. In diabetics, malonic acid, a polymer of the half aldehyde of malonic acid, or a condensation product of this half aldehyde might possibly be found after feeding propionic acid; but these products seem not to have been sought for.

Butyric acid *is partially oxidized only in the beta position.* Here too, this should be the most vulnerable position. A further oxidation at this point would lead finally to acetoacetic acid which by hydrolysis could lead either to acetone and carbon dioxide or to acetic acid. The increased excretion of acetoacetic acid and acetone after feeding butyric acid to diabetics is a well established fact.

The cited examples would lead one to conclude that the established fact that fatty acids oxidize predominantly in the beta position in the animal body, besides having a chemical verification is the fact that the same type of oxidation occurs with hydrogen peroxide, is exactly what one would expect from the electronic formulas of the fatty acids and their derivatives.

PART III.

Proof of the Electronic Formulas of a Few Biologically Important Organic Compounds.

The General Principles Used in the Determination of Electronic Formulas.

Principle I. The Polarity of a Double or Triple Bond.[15]—Consider an olefine derivative having the following structural formula:

$$A - \overset{H}{C} = \overset{H}{C} - B$$

[15] Falk, K. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1910, xxxii, 1637. Stieglitz has presented this matter in lecture form for the past 6 years, and has applied it to the derivation of the complete formulas for the vinyl derivatives, including acetaldehyde, and for the partial formulas of many organic compounds such as propylene, acrylic acid, crotonic acid, and cinnamic acid. The formula so derived for the vinyl derivatives is $H + - \underset{\underset{H}{+}}{\underset{-}{C}} \genfrac{}{}{0pt}{}{-+}{-+} \underset{\underset{H}{+}}{\underset{-}{C}} + - X$; for acetaldehyde is $H_3C - + \underset{\underset{H}{+}}{\underset{-}{C}} \genfrac{}{}{0pt}{}{+-}{+-} O$; for propylene is $H_3C - \underset{\underset{H}{+}}{\underset{-}{C}} \genfrac{}{}{0pt}{}{+-}{+-} \underset{\underset{H}{+}}{\underset{-}{C}} - + H$; for crotonic acid is $CH_3 - \underset{\underset{H}{+}}{\underset{-}{C}} \genfrac{}{}{0pt}{}{+-}{+-} \underset{\underset{H}{+}}{\underset{-}{C}} - COOH$; and for cinnamic acid is $C_6H_5 - \underset{\underset{H}{+}}{\underset{-}{C}} \genfrac{}{}{0pt}{}{+-}{+-} \underset{\underset{H}{+}}{\underset{-}{C}} - COOH$

The following three electronic formulas are possible.

$$A-\overset{H}{C}\begin{smallmatrix}+&-\\+&-\end{smallmatrix}\overset{H}{C}-B \qquad A-\overset{H}{C}\begin{smallmatrix}+&-\\-&+\end{smallmatrix}\overset{H}{C}-B \qquad A-\overset{H}{C}\begin{smallmatrix}-&+\\-&+\end{smallmatrix}\overset{H}{C}-B$$

Formula 1. Formula 2. Formula 3.

Which of these is the correct formula can be ascertained by determining how the olefine absorbs compounds of accepted polarity such as H+−OH, H+−Cl, H+−Br, H+−I, H+−NHR, and H+−OR.

A compound having formula (1) will absorb HBr, for example, to give

$$A-\begin{array}{c}H\\C\\+\\-\\Br\end{array}+-\begin{array}{c}H\\C\\-\\+\\H\end{array}-B \text{ exclusively.}$$

A compound having formula (2) will absorb HBr to give a mixture of

$$A-\begin{array}{c}H\\C\\-\\+\\H\end{array}+-\begin{array}{c}H\\C\\+\\-\\Br\end{array}-B \quad\text{and}\quad A-\begin{array}{c}H\\C\\+\\-\\Br\end{array}-+\begin{array}{c}H\\C\\-\\+\\H\end{array}-B$$

A compound having formula (3) will absorb HBr to give

$$A-\begin{array}{c}H\\C\\-\\+\\H\end{array}-+\begin{array}{c}H\\C\\+\\-\\Br\end{array}-B \text{ exclusively.}$$

Principle II. The Determination of the Charge on a Carboxyl Group.—Three methods have been employed.

1. In a few cases ketenes are known that have the general formula $R-C=C=O$. They always have the electronic formula $R-C\begin{smallmatrix}-&+\\-&+\end{smallmatrix}C\begin{smallmatrix}+&-\\+&-\end{smallmatrix}O$ because they yield acids, anilides, amines, and esters when treated with water, aniline, ammonia, and alcohol, respectively. This is an application of Principle I. In such cases the carboxyl group is quadruply positive.[16]

2. Many carboxylated compounds lose CO_2 when heated. Since CO_2 must have the formula $O\begin{smallmatrix}-&+\\-&+\end{smallmatrix}C\begin{smallmatrix}+&-\\+&-\end{smallmatrix}O$, such compounds contain a quadruply positive carboxyl group.[17]

[16] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1730.
[17] Fry, H. S., *J. Am. Chem. Soc.*, 1912, xxxiv, 664; 1914, xxxvi, 248.

3. Some carboxylated compounds split off formic acid when they are heated to 100–150° with dilute sulfuric acid.[18] Since formic acid must have the formula

$$\begin{array}{c} \mathrm{H} + - \mathrm{C} \substack{+\\+} \substack{-\\-} \mathrm{O} \\ \;\;\;\;\;+ \\ \;\;\;\;\;- \\ \;\;\;\;\;\mathrm{OH} \end{array}$$

the carboxyl group from which it was derived must be triply positive and it must have been attached to the neighboring carbon atom as follows:

$$\begin{array}{c} \mathrm{C} + - \mathrm{C} \substack{+\\+} \substack{-\\-} \mathrm{O} \\ \;\;\;\;\;+ \\ \;\;\;\;\;- \\ \;\;\;\;\;\mathrm{OH} \end{array}$$

4. The above carboxylated compounds give off carbon monoxide when they are warmed gently with fuming sulfuric acid. Carbon monoxide must have the formula $\pm \mathrm{C} \substack{+\\+} \substack{-\\-} \mathrm{O}$. The carbon is identical, electrically, with the carbon in formic acid. Its evolution has a significance identical with that ascribed to formic acid in the preceding paragraph.

A similar decomposition occurs with phosphoric acid.

The results are less reliable, however, because much higher temperatures are required to start the decomposition.

Principle III. Hydrolysis.—Frequent use has been made of the principle of hydrolysis which has been so thoroughly discussed by others[19] that a repetition seems superfluous.

Assumptions.

1. The charges on the carbon atoms are definitely polarized in at least the large majority of organic compounds.

There has been a tendency in recent years to assume that organic compounds are non-polar.[20] An entirely non-polar com-

[18] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1726.

[19] Nelson, J. M., Beans, H. T., and Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 1810. Selivanow, *Ber. chem. Ges.*, 1892, xxv, 3517. Stieglitz, J., *Am. Chem. J.*, 1896, xviii, 756. Noyes, W. A., *J. Am. Chem. Soc.*, 1913, xxxv, 769.

[20] For a criticism of this view see Falk, K. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1914, xxxvi, 209.

pound would be chemically inactive. It may, of course, be true that some or even most of the molecules are really non-polar but such molecules can be of no importance in initiating chemical reactions. *Such inactive non-polar molecules must always be in equilibrium with a certain number of active polar molecules. These active molecules are responsible for the chemical activity of the compound and they are very definitely polar.*[21] The present paper is limited to a discussion of the active polar molecules.

2. The electrical charges hold their places until they are forced to shift because of a definite overwhelming strain. Such shifts are infrequent, belong to classes of compounds rather than to individuals, and must never be assumed to have occurred unless this can be undeniably proved.[22]

References.

It will be necessary to refer frequently to the carbon atoms and to the bonds between the carbon atoms. Instead of placing numbers on the carbon atoms in each of the formulas, which would lead to confusion, the general rule has been adopted of numbering the carbon atoms mentally from *right* to *left* in a horizontal formula and from the top down in a vertical formula. A concrete example will make this clear. Crotonic acid has the structural formula $CH_3-CH=CH-COOH$. The carboxyl carbon is carbon atom 1, and the double bond is the (2–3) bond.

The facts, upon which the electronic formulas are based, can be verified by referring to any large handbook of organic chemistry such as Beilstein's Handbuch der Organischen Verbindungen and Richter's Organic chemistry.

A Consideration of the Formulas.

Propiolic Acid ($H-C\equiv C-COOH$).

When the potassium salt of this acid is heated with water it decomposes smoothly according to the following equation.

[21] Lewis, G. N., *J. Am. Chem. Soc.*, 1913, xxxv, 1448.

[22] Examples of such shifts can be found in the numerous papers by Stieglitz and his collaborators on the electronic interpretation of the Beckmann rearrangement.

$$2HC \equiv C - COOK + H_2O \rightarrow 2C_2H_2 + CO_2 + K_2CO_3$$

Since the carboxyl group is eliminated as CO_2, carbon atom 1 must be quadruply positive.

When propiolic acid is treated with the halogen acids, only the β-halogenated acrylic acids are formed. The exclusive formation of beta derivatives proves that the triple bond is unsymmetrically polar, the electrons being attached to carbon atom 2. The entire formula for propiolic acid, is, therefore,

$$H + - C \begin{smallmatrix}+&-\\+&-\\+&-\end{smallmatrix} C - + \underset{\substack{+\\-\\OH}}{C} \begin{smallmatrix}+&-\\+&-\end{smallmatrix} O \tag{1}$$

β-Halogenated Propiolic Acids ($X-C\equiv C-COOH$).

The halogenated propiolic acids decompose readily into $X-C\equiv C-H$ and CO_2. The carboxyl group is, therefore, quadruply positive.

The halogenated propiolic acids react with the halogen acids to give beta derivatives exclusively. Thus β-chloropropiolic acid gives β-dichloroacrylic acid with HCl. This proves that the triple bond is unsymmetrically polar so that the electrons are attached to carbon atom 2. The entire formula for this class of compounds is

$$X - + C \begin{smallmatrix}+&-\\+&-\\+&-\end{smallmatrix} C - + \underset{\substack{+\\-\\OH}}{C} \begin{smallmatrix}+&-\\+&-\end{smallmatrix} O \tag{2}$$

The carbon atoms in these acids are identical, electrically, with the carbon atoms in malonic acid.

Allylene ($CH_3-C\equiv C-H$).

Allylene is readily absorbed by the halogen acids, the products being $CH_3-CCl_2-CH_3$, $CH_3-CBr_2-CH_3$, and $CH_3-CI_2-CH_3$. In every case the negative halogen attaches itself to the central carbon atom. This unsymmetrical addition of the halogen acids proves that the triple bond is unsymmetrically polar, the electrons being attached to carbon atom 1.

When allylene is heated with dilute sulfuric acid, acetone is formed. Here again the negative oxygen attaches itself to the central carbon atom. Acetone has been proved[23] to have the formula

$$\begin{array}{ccccccc} & H & & & & H & \\ & + & & & & + & \\ & - & & & & - & \\ H+- & C & -+ & C & +- & C & -+H \\ & - & & ++ & & - & \\ & + & & -- & & + & \\ & H & & O & & H & \end{array}$$

Both of the end-carbon atoms are quadruply negative. The methyl group in methyl acetylene must, therefore, also be quadruply negative. The entire formula for methyl acetylene is

$$\begin{array}{cccccccc} & H & & & & & & \\ & + & & & & & & \\ & - & & & + & - & & \\ H+- & C & -+ & C & + & - & C & -+H \\ & - & & & + & - & & \\ & + & & & & & & \\ & H & & & & & & \end{array} \tag{3}$$

The carbon atoms in allylene are identical, electrically, with the carbon atoms in acetone.

Tetrolic Acid ($CH_3-C\equiv C-COOH$) and Its Relation to Acetoacetic Acid.

When tetrolic acid is heated to 210° it decomposes into allylene and carbon dioxide.

$$\begin{array}{ccccccccccccccccc} & & - & & & & & + & - & & & & & & & & \\ CH_3-C & & - & & C-COOH \rightarrow H_3C-+C & & & + & - & C-+H+O & - & + & C & + & - & O \\ & & - & & & & & + & - & & & - & + & & + & - & \end{array}$$

The complete electronic formula for tetrolic acid is, therefore,

$$\begin{array}{ccccccccccc} & H & & & & & & & & & \\ & + & & & & & & & & & \\ & - & & & + & - & & & & & \\ H+- & C & -+ & C & + & - & C & -+ & C & \begin{smallmatrix}+\\+\end{smallmatrix} & \begin{smallmatrix}-\\-\end{smallmatrix}\,O \\ & - & & & + & - & & & + & & \\ & + & & & & & & & - & & \\ & H & & & & & & & OH & & \end{array} \tag{4}$$

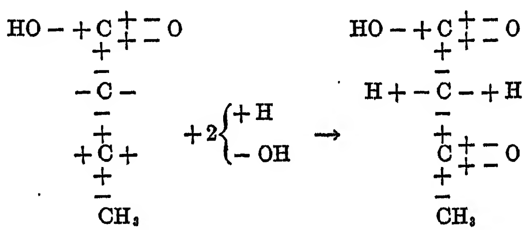
---

[23] See foot-note 2. This paper also contains the proof of the formulas for acetic, acetoacetic, acetone dicarbonic, and citric acids.

Since the above decomposition occurs at 210° it alone would not suffice to establish the formula for tetrolic acid. That the above formula is actually correct is proved by the following reactions for tetrolic acid.

It combines with the halogen acids to give β-halogenated tetrolic acids.

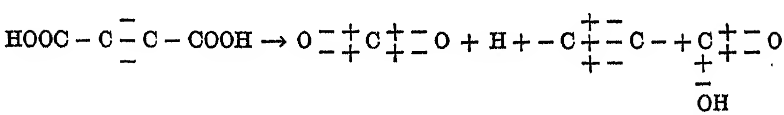
When heated to 105° with a concentrated solution of potassium hydroxide, it decomposes completely with the formation of large quantities of acetone and carbon dioxide and a small amount of acetic acid. This strongly suggests the intermediate formation of acetoacetic acid. This is to be expected from the assigned formula, because the carbon atoms in acetoacetic and in tetrolic acids are electronically identical.



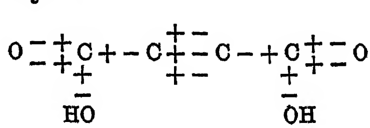
The acetoacetic acid would then give acetone and CO_2 in preponderance, and acetic acid in small amounts as is its habit.

Acetylene Dicarboxylic Acid (HOOC—C≡C—COOH).

When this acid is warmed with water it decomposes into propiolic acid and carbon dioxide.



Both carboxyl groups are quadruply positive. The formula for acetylene dicarboxylic acid is



(5)

Acrylic Acid ($CH_2 = CH-COOH$).

The recorded reactions for this substance are:

1. It unites with HCl and HI to give β-chloro- and β-iodo-propionic acid, respectively.

2. It gives β-oxypropionic acid, not lactic acid, when heated to 100° with an aqueous solution of sodium hydroxide.

3. When ethyl acrylate is heated with ethyl alcohol containing some sodium ethylate, β-ethoxypropionic acid is formed.

These three entirely one-sided reactions prove that the charges constituting the double bond are polarized so that the electrons are attached to carbon atom 2.

When potassium acrylate is fused with potassium hydroxide, hydrogen, potassium formate, and potassium acetate are formed. This reaction is obviously an hydrolysis at the double bond in which two positive hydrogen atoms are united with the alpha and two negative hydroxyl radicals with the beta carbon atom in exactly the manner that would be expected from the charges assigned to the double bond.

$$CH_2 = CH-COOK + H_2O \rightarrow H_2C = O + CH_3-COOK$$

The formaldehyde formed at first reacts in the customary manner with the potassium hydroxide to give hydrogen and potassium formate.

$$H_2C = O + KOH \rightarrow H_2 + HCOOK$$

The most important fact from our point of view, is the formation of acetic acid. The formula for this substance has been proved[23] to be

$$\begin{array}{ccccccc} & & H & & & & \\ & & + & & & & \\ & & - & & & & \\ H & +- & C & -+ & C & \begin{smallmatrix}+-\\+-\end{smallmatrix} & O \\ & & - & & + & & \\ & & + & & - & & \\ & & H & & OH & & \end{array}$$

It contains a quadruply positive carboxyl group. The carboxyl group in acrylic acid must, therefore, also be quadruply positive. This leads to the following complete formula for acrylic acid.

$$\begin{array}{ccccccccc} & & \mathrm{H} & & \mathrm{H} & & & & \\ & & + & & + & & & & \\ & & - & & - & & & & \\ \mathrm{H} & +- & \mathrm{C} & \substack{+\\+}\substack{-\\-} & \mathrm{C} & -+ & \mathrm{C} & \substack{+\\+}\substack{-\\-} & \mathrm{O} \\ & & & & & & + & & \\ & & & & & & - & & \\ & & & & & & \mathrm{OH} & & \end{array} \qquad (6)$$

Cinnamic Acid ($C_6H_5-CH \mathrel{=} CH-COOH$).

When cinnamic acid is treated with HBr or HI, β-halogenated phenylpropionic acids are formed exclusively. Hypochlorous acid is absorbed so that the negative hydroxyl group attaches itself to the beta carbon atom the product being phenyl α-chloro, β-lactic acid. *The exclusive formation of beta derivatives proves that the double bond* is unsymmetrically polar so that the electrons are attached to the alpha carbon atom.

When cinnamic acid is fused with potassium hydroxide, benzoic acid and acetic acid are the products, the negative OH group being *again*, at this high temperature, attracted *to the beta position*. The formation of acetic acid proves the carboxyl group to be quadruply positive. The formula for cinnamic acid is

$$\begin{array}{ccccccc} C_6H_5 - & \mathrm{C} & \substack{+\\+}\substack{-\\-} & \mathrm{C} & -+ & \mathrm{C} & \substack{+\\+}\substack{-\\-}\ \mathrm{O} \\ & - & & - & & + & \\ & + & & + & & - & \\ & \mathrm{H} & & \mathrm{H} & & \mathrm{OH} & \end{array}$$

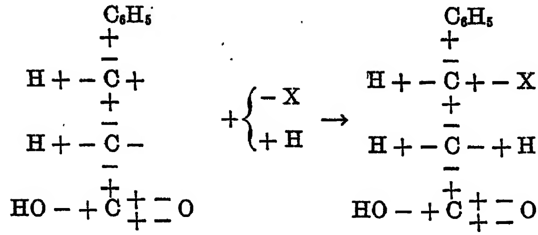
The remaining unsolved bond is probably (+−), because cinnamic acid gives ortho and para derivatives with HNO_3 exactly as is the case with phenol, aniline, and the halogenated benzenes in which the substituting group is surely negative. The entire formula for cinnamic acid would then be

$$\begin{array}{ccccccc} C_6H_5 +- & \mathrm{C} & \substack{+\\+}\substack{-\\-} & \mathrm{C} & -+ & \mathrm{C} & \substack{+\\+}\substack{-\\-}\ \mathrm{O} \\ & - & & - & & + & \\ & + & & + & & - & \\ & \mathrm{H} & & \mathrm{H} & & \mathrm{OH} & \end{array} \qquad (7)$$

It is electronically identical with acrylic acid, a positive C_6H_5 group having replaced the positive hydrogen.

β-Chloro-, Brom-, and Oxyphenylpropionic Acid ($C_6H_5-CHX-CH_2-COOH$).

The formulas for these substances follow as a natural corollary from that of cinnamic acid as can be seen from the following equation.



(8)

(8) is the formula for β-chloro-, brom-, or oxyphenylpropionic acid.

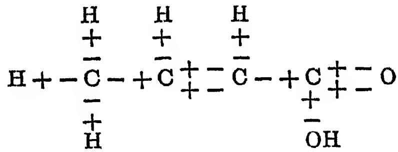
Crotonic Acid $(CH_3-CH=CH-COOH)$.

Crotonic acid combines with hydrogen iodide to give β-iodobutyric acid, with hydrogen bromide to give β-brombutyric acid and with sodium ethylate to give β-ethoxybutyric ester. In every case the negative radical attaches itself to the beta carbon atom. The double bond is, therefore, unsymmetrically polar, the electrons being attached to the alpha carbon atom.

When crotonic acid is fused with potassium hydroxide it is hydrolyzed at the double bond to give acetaldehyde and acetic acid. The acetaldehyde formed at first is not liberated as such but reacts with KOH in the customary manner to give acetic acid and hydrogen.

$$CH_3-CH=CH-COOK + KOH \rightarrow CH_3-CHO + CH_3-COOK$$
$$CH_3-CHO + KOH \rightarrow CH_3-COOK + H_2$$

At high temperatures the double bond is still unsymmetrically polar so that the negative OH groups attach themselves to the beta carbon atom. The important fact is the formation of 2 molecules of acetic acid because this proves the polarity of the (1–2) and the (3–4) bond. Acetic acid contains a quadruply positive carboxyl group; so the carboxyl group in crotonic acid must also be quadruply positive. The second molecule of acetic acid, coming as it does from the acetaldehyde formed at first, proves that the (3–4) bond must have an electrical polarity like that of acetaldehyde; namely, (C−+C). The entire formula for crotonic acid is



(9)

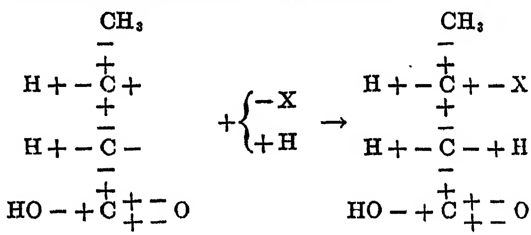
Crotonic acid contains the acrylic acid nucleus.

β-Oxy- and β-Halogenated Butyric Acids ($CH_3-CHX-CH_2-COOH$).

The facts that are needed for the proof of these formulas have already been given under crotonic acid. They will be repeated here for the sake of clearness.

When crotonic acid is heated with an aqueous solution of sodium hydroxide, β-oxybutyric acid is formed.

When crotonic acid is heated with hydrogen bromide or iodide, β-brom- and β-iodobutyric acids are formed, respectively. Electronically these reactions proceed as follows.



(10)

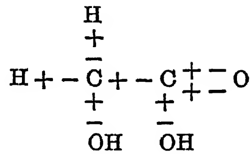
Formula (10) is that of β-oxy, β-ethoxy, and β-halogenated butyric acids. The carbon atoms are electrically identical with the carbon atoms in crotonic acid. It is not surprising, then, to find that they readily lose HX to give crotonic acid.

Glycollic Acid ($CH_2OH-COOH$).

When glycollic acid is warmed with concentrated sulfuric acid it decomposes into carbon monoxide, water, and trioxymethylene.

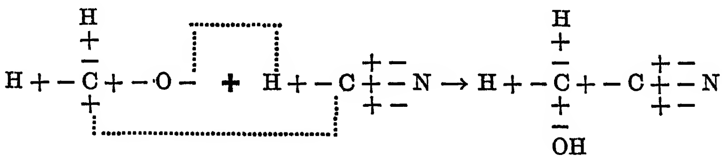
$$3\ CH_2OH-COOH \rightarrow 3CO + 3H_2O + (CH_2O)_3$$

In this case the carboxyl group is split off as carbon monoxide and the formaldehyde formed at first polymerizes to trioxymethylene. The carboxyl group must, therefore, be triply positive. The complete formula for glycollic acid is



(11)

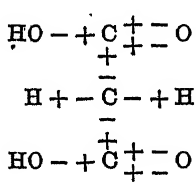
This formula is substantiated by the preparation of glycollic acid from formaldehyde and prussic acid.



The nitrile then gives the acid on hydrolysis which can produce no change in the electrical conditions of the molecule.

Malonic Acid ($COOH-CH_2-COOH$).

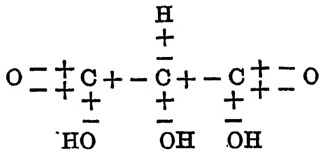
When the acid is heated above its melting point, 132°, it decomposes smoothly into CO_2 and acetic acid. The evolution of CO_2 proves that at least *one* of the carboxyl groups is quadruply positive. Acetic acid has been proved to contain a positive carboxyl group; hence the second carboxyl group in malonic acid must also be quadruply positive. The entire formula for this substance is, therefore,



(12)

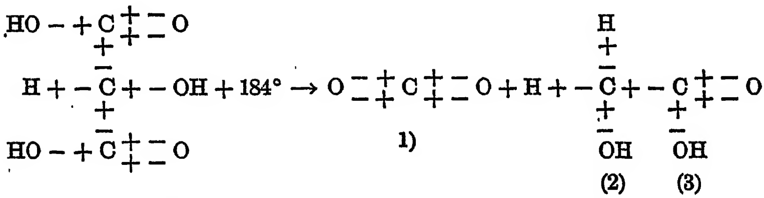
Tartronic Acid ($HOOC-CHOH-COOH$).

When tartronic acid is heated above its melting point, 184°, it decomposes smoothly into carbon dioxide and a polymer of glycollic acid. The evolution of CO_2 proves that *one* of the carboxyl groups is quadruply positive. Glycollic acid has been proved to have a negative carboxyl group. The formula for tartronic acid must then be



(13)

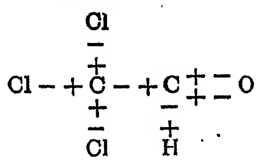
The decomposition can be represented electronically as follows



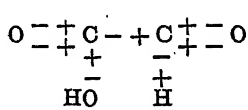
Mesoxalic Acid ($HOOC-CO-COOH$).

When this acid is heated above its melting point it decomposes into carbon dioxide and glyoxylic acid. The evolution of CO_2 proves that one of the carboxyl groups is quadruply positive. The formula for glyoxylic acid cannot be proved from the recorded reactions of that substance; but the following indirect proof seems rather convincing.

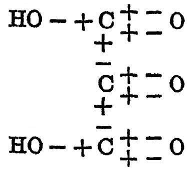
The formula for chloral must be



because it gives chloroform and sodium formate when treated with aqueous alkalies. If it were possible to substitute OH groups for the three negative halogen atoms without separating the carbon atoms, glyoxylic acid would be formed. The formula for glyoxylic acid should then be



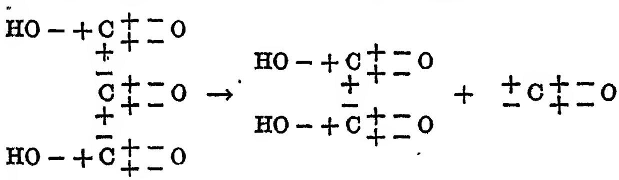
From this one would conclude that one of the carboxyl groups in mesoxalic acid was triply positive and that the entire formula for this substance is



(14)

One assumption has been made; namely, that the substitution of negative oxygen for negative chlorine in chloral has produced no change in the polarity of the charges binding the carbon atoms together.

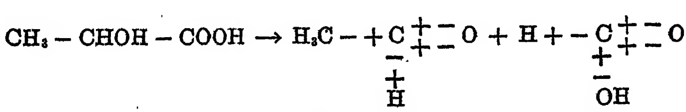
That we were justified in making the above assumption, and that the formula assigned to mesoxalic acid is really correct is proved in a striking manner by the fact that the acid decomposes on boiling with water into carbon monoxide and oxalic acid. Electronically this reaction would be represented as follows.



The carbon monoxide might arise from either the central or the triply positive end-carbon atom. In any case *it would be impossible to obtain CO and oxalic acid from a molecule having any but the above electronic structure.*

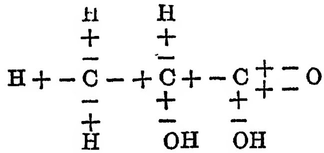
Lactic Acid ($CH_3-CHOH-COOH$).

When lactic acid is treated at 60° with fuming sulfuric acid, the carboxyl group is eliminated as carbon monoxide. A similar decomposition occurs when lactic acid is heated to 130° with dilute sulfuric acid, the products being acetaldehyde and formic acid.



(3) (2) (1)

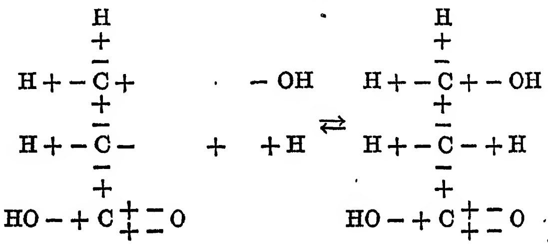
From the above equation the formula for lactic acid is easily seen to be



(15)

β-Oxypropionic Acid (CH_2OH-CH_2-COOH).

When acrylic acid is heated to 100° with an aqueous solution of sodium hydroxide, β-oxypropionic acid is formed. Electronically, this reaction can be written as follows.

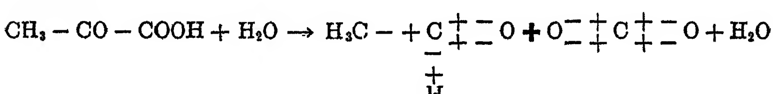


(16)

The carbon atoms in β-oxypropionic acid are identical, electronically, with those in acrylic acid. It is not surprising to find, then that β-oxypropionic acid readily loses water to give acrylic acid. To obtain acrylic acid from *lactic acid*, a far reaching electronic rearrangement is necessary and one would expect that to bring about this transformation, a more drastic treatment would be required. This is the case. The difference in ease of activity between the α- and β-oxy (and halogenated) derivatives is readily explained, in this manner by the electronic formulas.

Pyruvic Acid ($CH_3-CO-COOH$).

When pyruvic acid is heated to 150° with dilute sulfuric acid it is hydrolyzed, the products being acetaldehyde and carbon dioxide.



(3) (2) (1)

From the above partially electronic equation the entire formula for pyruvic acid is readily seen to be

$$\begin{array}{ccccccc}
 & & \mathrm{H} & & & & \\
 & & + & & & & \\
 & & - & & & & \\
\mathrm{H}\,+ & - & \mathrm{C} & -\;+ & \mathrm{C} & -\;+ & \mathrm{C}\,\begin{smallmatrix}+\\+\end{smallmatrix}\,\begin{smallmatrix}-\\-\end{smallmatrix}\,\mathrm{O} \\
 & & - & & ++ & & + \\
 & & + & & -\,- & & - \\
 & & \mathrm{H} & & \mathrm{O} & & \mathrm{OH}
\end{array} \tag{17}$$

Both pyruvic and oxalic acids contain the electrical dyad $\left(\begin{smallmatrix}+\\+\\+\end{smallmatrix}\mathrm{C} - + \mathrm{C}\begin{smallmatrix}+\\+\\+\end{smallmatrix}\right)$. They are similarly hydrolyzed by sulfuric acid.

Malic Acid ($HOOC-CH_2-CHOH-COOH$).

When malic acid is heated with dilute sulfuric acid, it decomposes into CO_2, formic acid, and acetaldehyde. When malic acid is warmed with fuming sulfuric acid it decomposes quantitatively according to the following equation.[24]

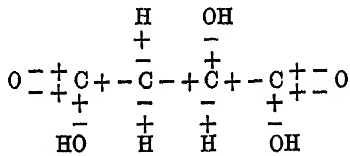
$$\begin{array}{llcl}
HOOC-CH_2-CHOH-COOH \rightarrow & HOOC-CH_2-CHO & (A) & +\ HCOOH \\
 & \quad\downarrow & & \quad\downarrow \\
 & C_5H_4O_2-COOH & (B) & CO+H_2O
\end{array}$$

Compound (B) is a polymer of (A). When (B) is boiled with dilute sulfuric acid it decomposes into CO_2 and acetaldehyde. Although compound (A) has never been isolated—it polymerizes too readily—its formation as an intermediate product in the decomposition of malic acid by sulfuric acid is proved conclusively by the character of the condensation products formed when malic acid, sulfuric acid, and phenols are heated together.

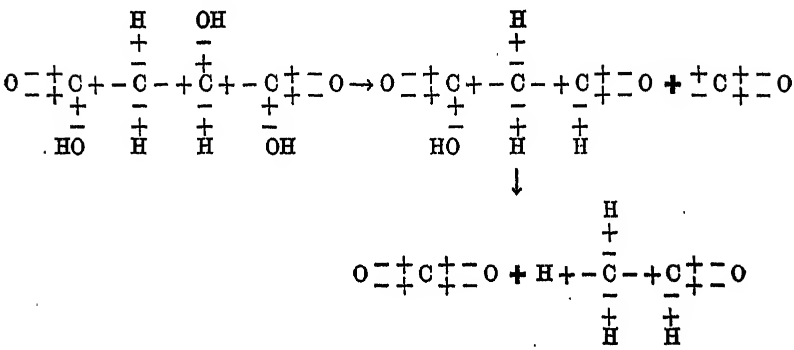
This reaction proves the entire formula for malic acid. Since CO is evolved—or formic acid with dilute sulfuric acid—*one* of the carboxyl groups must be negative. The negative carboxyl group is attached to carbon atom 1 (see the above equation). The evolution of CO_2 from the polymer of $HOOC-CH_2-CHO$ proves that the other carboxyl group is quadruply positive. The other product is acetaldehyde, a compound of known electronic constitution (CH_3-+CHO).

[24] von Pechmann, H., and Welsh, W., *Ber. chem. Ges.*, 1884, xvii, 929, 1649.

The entire formula for malic acid is

 (18)

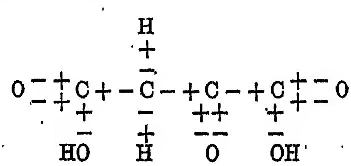
This proof is rendered clear by expressing the decomposition electronically.



Oxalacetic Acid ($HOOC-CH_2-CO-COOH$).

The formula for this substance is clearly proved by the following decompositions of its diethyl ester. When boiled with alkalies it is hydrolyzed so that the products are oxalic acid, acetic acid, and alcohol. When boiled with dilute acids it is hydrolyzed so that carbon dioxide and pyruvic acid are formed. When heated under ordinary pressures it decomposes into carbon monoxide and malonic ethyl ester.

Only one formula can be written that readily explains all of these decompositions; namely,

 (19)

As can be seen, an hydrolysis at bond (2–3) would give acetic and oxalic acids; at bond (3–4) would give carbon dioxide and pyruvic acid. The loss of carbon atom 2 would give carbon monoxide and malonic acid.

Tartaric Acid (HOOC−CHOH−CHOH−COOH).

Tartaric acid reacts readily with concentrated sulfuric acid or phosphoric acid. The several observers seem to have obtained different products when operating under slightly different conditions. Thus Vangel[25] found that when tartaric acid is heated to 150° with phosphoric acid, equal volumes of CO_2 and CO were formed. He did not examine the non-gaseous residue, nor did he measure the exact quantity of gas obtained per unit weight of tartaric acid. Bouchardat[26] found that when tartaric acid is warmed to 40–50° with fuming sulfuric acid containing 80 per cent of SO_3, a gas is formed that is composed of 4 parts of CO and 1 part of SO_2, and which contains from 2 to 4 per cent of carbon dioxide. He states, moreover, that the carbon dioxide and SO_2 appeared toward the *end* of the reaction. An examination of the non-gaseous residue revealed the presence of some racemic tartaric acid and small amounts of glycollic and pyruvic acids. He gives no data by means of which one could calculate how many of the carbon atoms of tartaric acid were evolved as carbon monoxide. Since these data were necessary to establish the electronic formula for tartaric acid, the following experiment was carried out.

Tartaric acid—1.5000 gm., M.P. 170°—was mixed in a 50 cc. round bottomed flask with 25 cc. of fuming sulfuric acid containing 18 per cent of SO_3. The flask was connected to a 1,000 cc. narrow mouthed precision cylinder that had been inverted and arranged so that the evolved gases could be collected by displacement of water. The flask was heated to 65°. A gas was slowly and steadily evolved that was pure carbon monoxide at first. Not a trace of either SO_2 or CO_2 was present until the gas volume, corrected for temperature, pressure, and aqueous tension was 445 cc. At this point the evolution of gas becomes livelier.

[25] Vangel, B., *Ber. chem. Ges.*, 1880, xiii, 356.
[26] Bouchardat, M. G., *Bull. Soc. chim.*, 1880, xxxiv, series 2, 495.

Considerable SO_2 was present as evidenced by the white fumes that formed when the gas came in contact with the wet walls of the cylinder. The reaction was practically over when about 950 cc. of gas had been collected. The volume shrunk to 840 cc. on agitation with water. This removed all of the SO_2 and probably also some of the CO_2. A subsequent agitation with aqueous sodium hydroxide gave a final volume of 800 cc. of gas that proved to be pure carbon monoxide.

The sulfuric acid was colored very pale yellow after the reaction was over so that no charring had occurred.

An examination of this reaction shows the following. Carbon monoxide (445 cc.) was evolved from 1.5 gm. of tartaric acid before either SO_2 or CO_2 were formed. This amount of CO was liberated by the primary action of the sulfuric acid before an oxidation reaction had occurred. This quantity of tartaric acid should give about 225 cc. of CO per carbon atom or 900 cc. if all of the carbon atoms had been converted into CO. The 445 cc. actually obtained represent two carbon atoms, half of the tartaric acid molecule. Since two of the four carbon atoms were evolved as CO *before an oxidation reaction had occurred*, it is fairly safe to conclude that *both* of the *carboxyl groups* were eliminated as CO according to the following equation

$$(CHOH)_2\,(COOH)_2 \rightarrow 2CO + CHO - CHO + 2H_2O$$

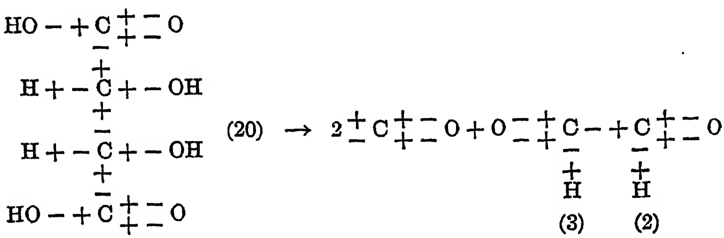
Both of the carboxyl groups must, therefore, have been triply positive. The assumption that glyoxal is an intermediate product in the decomposition of tartaric acid by fuming sulfuric acid is in perfect agreement with the observed fact that all of the carbon atoms in tartaric acid are finally evolved as CO. A primary oxidation of glyoxal would yield glyoxylic acid which would then be converted largely into CO and to a small extent into oxalic acid, CO, and CO_2.

$$1.\left\{\begin{array}{lll} CHO - CHO + H_2SO_4 & \rightarrow & CHO - COOH + SO_2 + H_2O \\ CHO - COOH & \rightarrow & HCOOH \quad + CO \\ HCOOH & \rightarrow & CO \quad + H_2O \end{array}\right.$$

$$2.\left\{\begin{array}{lll} CHO - COOH + H_2SO_4 & \rightarrow & COOH - COOH + SO_2 + H_2O \\ COOH - COOH & \rightarrow & CO \quad + CO_2 \end{array}\right.$$

The evolution of so much CO and so little CO_2 could not be explained at all if the assumption were made that even one of the carboxyl groups in tartaric acid was quadruply positive.

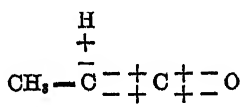
The complete electronic formula for tartaric acid and its primary decomposition into CO, glyoxal, and water can be represented as follows.



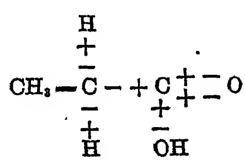
(20)

Propionic Acid (CH_3-CH_2-COOH).

That the carboxyl group in propionic acid is quadruply positive is proved by the absorption reactions of methyl ketene, $CH_3-CH \overset{-}{\underset{+}{=}} C \overset{-}{\underset{+}{=}} O$. Methyl ketene unites with water to give propionic acid, with alcohol to give ethyl propionate, and with ammonia to give propionamide. In every case the negative radical attaches itself to carbon atom 1. This proves that the double bond is unsymmetrically polar; that the partial formula for methyl ketene is

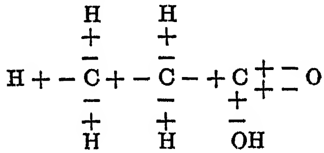
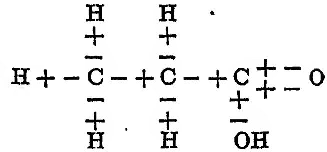


and that propionic acid has the partial formula



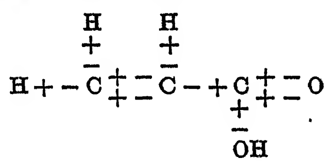
The polarity of the remaining unsolved bond cannot be determined directly. The following partially speculative proofs seem convincing to the authors.

Just two formulas are possible, namely,

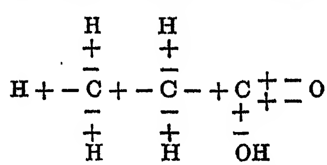
(1)  (2) 

It is, of course, possible that both of these formulas are correct and that the two electrons between carbon atoms 2 and 3 are vibrating so that they are attached first to one and then to the other carbon atom (or they may hold an electron in common). Although the possibility of such an electrometric equilibrium is undeniable, we have no right to assume its existence without proof. Most of the simple compounds examined so far have shown a rigid polarity. In the absence of proof to the contrary we will assume that the same rigidity exists in this compound. There are two proofs that formula (1) is representative of the electrical conditions existing in the molecule of propionic acid.

1. Acrylic acid has been proved to have the formula



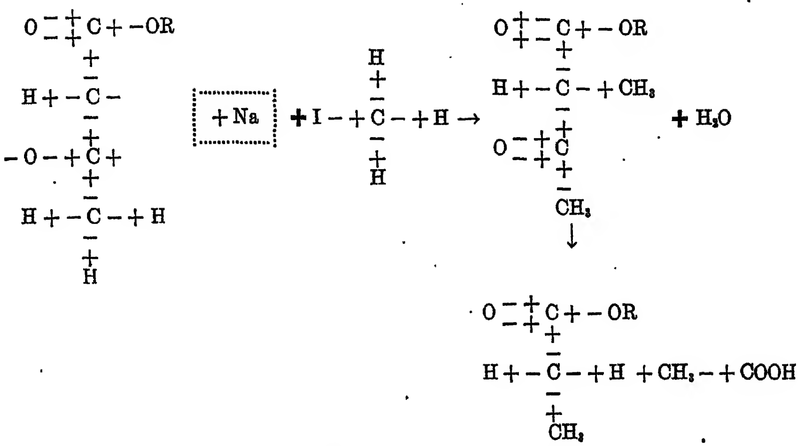
By reduction acrylic acid gives propionic acid. The reduction consists in the addition of two electrons to the beta carbon atom. The formula for propionic acid should then be



(21)

One assumption must be made; namely, that an electronic shift does not take place *within the molecule after the electrons and the hydrogen atoms have been added.*

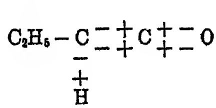
2. Propionic acid can be prepared from the sodium compound of acetoacetic or malonic ester and methyl iodide. The reactions can be represented electronically as follows.



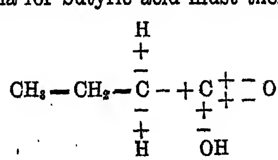
This formula is also obtained by the similar reaction of methyl iodide with the sodium compound of malonic ester and is identical with formula (1).

Butyric Acid ($CH_3-CH_2-CH_2-COOH$).

The carboxyl group in butyric acid is quadruply positive. This can be proved from the absorption reactions of ethyl ketene ($CH_3-CH_2-CH\overset{+}{\underset{-}{=}}C\overset{+}{\underset{-}{=}}O$). Ethyl ketene absorbs water, alcohol, and aniline so that the negative OH, OC_2H_5, and $NH-C_6H_5$ groups attach themselves to carbon atom 1, the products being butyric acid, ethyl butyrate, and butyric acid anilide. These entirely one-sided reactions prove that the double bond in ethyl ketene is unsymmetrically polar so that the electrons are attached to carbon atom 2. The partial formula for ethyl ketene is, therefore,



The partial formula for butyric acid must then be



The remaining bonds can be solved only by speculation as follows. Crotonic acid has been proved to have the formula

$$H_3C - +\underset{\underset{\underset{H}{+}}{-}}{C}\,{}^{+}_{+}\,{}^{-}_{-}\,\underset{\underset{\underset{H}{+}}{-}}{C} - +\underset{\underset{\underset{OH}{-}}{+}}{C}\,{}^{+}_{+}\,{}^{-}_{-}\,O$$

By reduction, crotonic acid gives butyric acid. The reduction consists in the addition of two electrons to the beta carbon atom. The formula for butyric acid should then be

$$H + -\underset{\underset{\underset{H}{+}}{-}}{\overset{\overset{H}{+}}{\overset{-}{C}}} - +\underset{\underset{\underset{H}{+}}{-}}{\overset{\overset{H}{+}}{\overset{-}{C}}} + -\underset{\underset{\underset{H}{+}}{-}}{\overset{\overset{H}{+}}{\overset{-}{C}}} - +\underset{\underset{\underset{OH}{-}}{+}}{C}\,{}^{+}_{+}\,{}^{-}_{-}\,O \qquad (22)$$

Although this must certainly be the formula at the moment of reduction, the objection might be raised that the electrical charges could shift after the hydrogen atoms and the electrons had been added. To assume that such a shift did occur would be unreasonable unless the molecule so formed contained electrical dyads of uncertain stability. On the other hand, if the certain stability of every dyad in the molecule could be proved, the above formula should be considered correct until some definite proof to the contrary was found. The undoubted stability of every dyad in this formula is proved by considering the electronic skeleton of the acrylic acid molecule.

$$\underset{\text{(Acrylic acid.)}}{+\underset{-}{\overset{-}{C}}+ -\underset{-}{\overset{-}{C}}- +\underset{+}{\overset{+}{C}}+} \qquad \underset{\text{(Butyric acid.)}}{-\underset{-}{\overset{-}{C}}- +\underset{-}{\overset{-}{C}}+ -\underset{-}{\overset{-}{C}}- +\underset{+}{\overset{+}{C}}+}$$

The dyad $+\underset{-}{\overset{-}{C}}+ -\underset{-}{\overset{-}{C}}-$ appears once in the electrically very stable acrylic acid and twice in the assigned formula for butyric acid. The formula assigned to butyric acid is, therefore, not only possible but extremely probable.

Phenylpropionic Acid ($C_6H_5-CH_2-CH_2-COOH$).

The method used in proving the formula for phenylpropionic acid is almost identical with that just described for propionic

acid. A detailed proof is withheld to avoid unnecessary repetition. The formula is

$$\begin{array}{ccccccccc}
 & & H & & H & & & & \\
 & & + & & + & & & & \\
 & & - & & - & & & & \\
C_6H_5 & +- & C & +- & C & -+ & C & \genfrac{}{}{0pt}{}{+-}{+-} & O \\
 & & - & & - & & + & & \\
 & & + & & + & & - & & \\
 & & H & & H & & OH & &
\end{array} \qquad (23)$$

The charge on the benzene ring is given as positive because phenylpropionic acid gives ortho and para derivatives just as is the case with all of the negatively mono-substituted derivatives of benzene. Phenylpropionic acid is electronically identical with propionic acid, a positive hydrogen atom having been replaced by a positive phenyl group.

Two New Electronic Principles.

Principle IV.—The polarity of the double bond in an unsaturated aliphatic compound is determined by the electrical charge on the first substituting group.

An examination of the formulas just presented has led us to conclude that the polarity of the double bond in an unsaturated aliphatic compound is determined by the electrical charge on the first substituting group which we will call the directing group.

There are two sharply defined types of unsaturated compounds, those belonging to the *vinyl type*

$$H_2C \genfrac{}{}{0pt}{}{-+}{-+} \underset{H}{C} +- V$$

and those belonging to the *acrylic acid type*

$$H_2C \genfrac{}{}{0pt}{}{+-}{+-} \underset{H}{C} -+ A$$

When the first substituent is negative the compound belongs to the vinyl type. Such a compound will always give alpha derivatives by the absorption of a polar compound like HCl. When the first substituent is positive the compound belongs to the acrylic acid type; it gives only beta derivatives by the absorption of polar compounds like HCl.

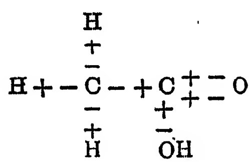
Beside these two simple types, one very stable and common mixed type is possible; namely, $V-+C \genfrac{}{}{0pt}{}{+-}{+-} C-+A$. A com-

pound belonging to this type is both a vinyl and an acrylic acid derivative. Such compounds are electrically very stable because their polarity is fixed by two groups both of which are striving to produce the same electrical configuration.

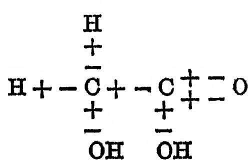
Of the compounds discussed so far, allylene belongs to the vinyl type, propiolic acid to the acrylic acid type, and crotonic, cinnamic, tetrolic, and the β-halogenated propiolic acids belong to the mixed type. The chemical properties of many unsaturated compounds are too incompletely known to make it possible to prove their electronic formulas completely. In such cases Principle IV is frequently of service because it gives us a means of writing a fairly authentic formula on the basis of a few absorption reactions. Examples of such usage will be given presently.

Principle V.—A carbon atom that is attached to an oxygen atom will become at least doubly positive, when this is at all possible.

Glycollic acid is the simplest example of the truth of this principle. The electronic formula for acetic acid is



When one of the paraffin hydrogen atoms is replaced by an OH group, the compound formed, glycollic acid, has the formula



The introduction of one OH group has caused carbon atom 2 to lose *four* electrons. From this it would seem that a triply negative carbon atom is electrically unstable when it is combined with oxygen. The triply negative carbon atom will, when this is possible, lose two electrons to a neighboring carbon atom. Of the compounds discussed so far, citric, tartronic, lactic, malic, and tartaric acids can be cited as illustrations of the truth of this principle. All of these compounds are α-hydroxy-acids and

they all contain a negative carboxyl group. In all of these cases, the alpha carbon atom, which is united with the OH group, is at least doubly positive.

The real value of Principles IV and V lies in the fact that they give us a means of accurately predicting the behavior of certain classes of organic compounds and of establishing the formulas for some compounds whose chemical properties are too incompletely known to make a complete proof of their electronic formulas possible. We will now consider a few such examples.

Propylene ($CH_2{=}CH-CH_3$).

In all of its absorption reactions, propylene gives only isopropyl derivatives. Thus HCl, HBr, and HI give isopropyl chloride, bromide, and iodide respectively. Sulfuric acid gives isopropyl sulfate which by hydrolysis gives isopropyl alcohol. This proves that the electrical charges constituting the double bond are unsymmetrically polar, the electrons being attached to carbon atom 3.

$$\begin{array}{ccccc} H+- & C & \begin{smallmatrix}-&+\\-&+\end{smallmatrix} & C & -CH_3 \\ & - & & - & \\ & + & & + & \\ & H & & H & \end{array}$$

The polarity of the remaining unsolved bond cannot be determined directly. That the CH_3 group carries a negative charge is rendered highly probable by the following line of reasoning.

The polarity of the double bond in propylene is like that in the vinyl compounds. Propylene must, then, be a vinyl derivative; hence by Principle IV, the methyl group is negative. The complete formula for propylene is

$$\begin{array}{ccccc} H+- & C & \begin{smallmatrix}-&+\\-&+\end{smallmatrix} & C & +-CH_3 \\ & - & & - & \\ & + & & + & \\ & H & & H & \end{array} \tag{24}$$

That an unopposed methyl group normally carries a negative charge is clearly shown by an examination of some aromatic and aliphatic compounds. Toluene gives ortho and para derivatives almost exclusively just as is the case with phenol and chlorobenzene. The methyl group, like the OH and Cl radicals, functions negatively. Negative methyl groups have also been encountered

in acetic acid, acetone, acetoacetic acid, tetrolic acid, allylene, acetone dicarbonic acid, and citric acid. Propionic acid is the only compound that we have encountered up to date that *may* contain a positive methyl group.

Allyl Alcohol ($CH_2{=}H-CH_2OH$).

When allyl alcohol is heated with a saturated solution of potassium acid sulfite, $CH_2SO_3H-CH_2-CH_2OH$ is formed, the negative SO_3H group going to the beta carbon atom.

When allyl alcohol is treated with hypochlorous acid, $CH_2OH-CH_2Cl-CH_2OH$ is formed, the negative OH group attaching itself to the beta carbon atom.

This proves that the double bond in allyl alcohol is unsymmetrically polar so that the electrons are attached to carbon atom 2. The charge on the CH_2OH group cannot be determined directly; but there is good evidence that it is positive and that the entire formula for allyl alcohol is

$$\begin{array}{ccccccccccc}
 & & & H & & H & & & H & & \\
 & & & + & & + & & & + & & \\
 & & & - & & - & & & - & & \\
H & + & - & C & \substack{+\,-\\+\,-} & C & - & + & C & + & OH \\
 & & & & & & & & - & & \\
 & & & & & & & & + & & \\
 & & & & & & & & H & &
\end{array} \qquad (25)$$

The indirect proof of this statement is as follows.

1. Allyl alcohol gives only beta derivatives when it absorbs electrically polar compounds. In this respect it behaves exactly like acrylic acid, and it belongs, therefore, to the acrylic acid type, *not* to the vinyl type. The CH_2OH group should, therefore, be positive like the carboxyl group in acrylic acid (Principle IV).

2. By Principle V, a carbon atom that is attached to oxygen will become at least doubly positive. The oxymethyl group in allyl alcohol should, therefore, be positive.

3. To assume that the oxymethyl group was negative would be unreasonable, because if that were true, the polarity of the double bond in allyl alcohol and in propylene should be identical. We can see no reason why an added oxygen atom should change the *remote* electrical charges on the molecule if it were incapable of changing the charge on the carbon atom to which it is attached.

Allyl Chloride $(CH_2{=}CH-CH_2Cl)$.

Allyl chloride combines with hydrogen chloride at 100° to give propylene chloride, $CH_3-CHCl-CH_2Cl$. Concentrated sulfuric acid is readily absorbed by allyl chloride at room temperatures. When the resulting acid sulfate is treated with water, the product is $CH_3-CHOH-CH_2Cl$. The negative group attaches itself to carbon atom 2 in each case. These facts prove that for temperature ranges from 20 to 100° the charges constituting the double bond are unsymmetrically polar, *the electrons being attached to the beta carbon atom.* The partial formula for allyl chloride is

$$H+-\underset{\underset{H}{+}}{\underset{-}{C}}\;\substack{-\\-}\,\substack{+\\+}\;\overset{\overset{H}{+}}{\overset{-}{C}}-CH_2Cl$$

The charge on the CH_2Cl group cannot be determined directly; but there is good evidence that it is negative and that the complete formula for allyl chloride is

$$H+-\underset{\underset{H}{+}}{\underset{-}{C}}\;\substack{-\\-}\,\substack{+\\+}\;\overset{\overset{H}{+}}{\overset{-}{C}}+-\underset{\underset{H}{+}}{\underset{-}{\overset{\overset{H}{+}}{\overset{-}{C}}}}+-Cl \qquad (26)$$

The absorption reactions of allyl chloride are identical with those of propylene and the vinyl derivatives but the reverse of those of acrylic acid and allyl alcohol. Allyl chloride is, therefore, a vinyl derivative; the charge on the CH_2Cl group is negative. The CH_2OH group has just been shown to be *positive.* Although the negative oxygen atom will, when possible, repel two electrons from the carbon atom to which it is attached, this seems not to be the case with the negative chlorine atom. Stated differently, when a carbon atom is attached to an oxygen atom the carbon atom will be charged ${}^{+}_{-}C^{+}_{-}$ in preference to ${}^{-}_{-}C^{+}_{-}$, the latter form being rarely capable of existence. *A carbon atom that is attached to chlorine is perfectly stable when it is charged*

$\overset{+}{\underset{-}{\bar{}C}}$. This would indicate that triply negative carbon is not *inherently* unstable; it is the attached oxygen atom that renders it so.

The negative charge assigned to the CH_2Cl group is in perfect agreement with its directing force as manifested in the aromatic series. Benzyl chloride gives ortho and para derivatives on halogenation and nitration just as is the case with all of the negatively mono-substituted benzene derivatives.

It might not be out of place at this point to call attention to the need of certain facts that would help greatly to establish the electronic formulas of some biologically very important compounds.

1. Since the CH_2OH group in allyl alcohol is surely positive, it ought also to be positive in benzyl alcohol. Then benzyl alcohol or any of the ethers derived from it should direct the second incoming substituent predominantly to the meta position.

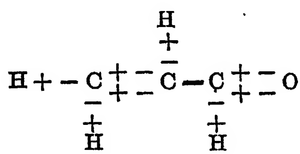
2. It is very important for protein chemistry that we know how the NH_2 group affects the carbon atom to which it is attached. Triply negative carbon has been shown to be stable when attached to chlorine and unstable when attached to oxygen. Although $CH_2OH+-COOH$ is surely the formula for glycollic acid, $CH_2Cl-+COOH$ is probably, therefore, the formula for monochloroacetic acid. What is the formula for aminoacetic acid? This will be hard to prove directly because glycine is an extremely stable compound; but the determination of the following facts would render an indirect proof possible. Does allyl amine give alpha or beta derivatives when it is treated with polar compounds? Does benzyl amine give ortho and para or meta derivatives? From these facts it would be possible to conclude whether the NH_2 group repelled electrons from the carbon atom to which it is attached as is the case with the OH group, or whether triply negative carbon is stable when attached to the NH_2 group as it is when attached to chlorine.

3. Does oxygen repel electrons from the carbon atom to which it is attached *because of its strongly negative character?* This could be determined by examining the properties of certain fluorine derivatives. Since fluorine is more decidedly negative than oxygen, it should be able to repel electrons from the carbon atom to which it is attached at least as certainly as oxygen *if this repulsion was occasioned by the negative character of the fluorine atom.* The oxy-

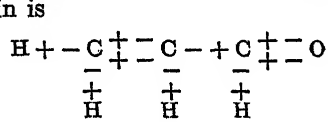
gen atom may prove to be unique in this respect, which might throw some light upon the intra-atomic structure of this atom. The absorption reactions of allyl fluoride and the behavior of benzyl fluoride on nitration, etc., would settle this point.

Acrolein $(CH_2\overset{-}{=}CH-CHO)$.

When acrolein is treated at low temperatures with hydrogen chloride or hydrogen bromide, β-chloro- and β- brompropionaldehyde are formed respectively. This proves that the electrical charges constituting the double bond are unsymmetrically polar, the electrons being attached to carbon atom 2. The partial formula for acrolein is



The charge on the aldehyde group cannot be determined directly; but there is good evidence that it is positive and that the complete formula for acrolein is



(27)

1. The absorption reactions of acrolein are identical with those of acrylic acid and allyl alcohol; but the reverse of those of the vinyl derivatives. Acrolein, therefore, belongs to the acrylic acid type; the charge on the aldehyde group is positive (Principle IV).

2. Since the charge on the carboxyl group of acrylic acid has been proved to be positive and that on the oxymethyl group of allyl alcohol was proved to be most probably positive, it would be strange indeed if the aldehyde group, which stands between the CH_2OH and the COOH groups in degree of oxidation, should be negative.

3. Aldehyde groups are usually if not invariably positive. In glyoxylic acid, for example, where an aldehyde and a carboxyl group vie for the electron, it is the aldehyde group that is positive. In the aromatic series the aldehyde group directs the second incoming substituent predominantly to the meta position as is the case with nitrobenzene.

STUDIES ON PROTEINOGENOUS AMINES.

XIV. A MICROCHEMICAL COLORIMETRIC METHOD FOR ESTIMATING TYROSINE, TYRAMINE, AND OTHER PHENOLS.

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INTRODUCTION.

The discovery of the diazonium salts by Griess in 1866[1] opened up a most fruitful field for scientific investigation by giving the investigator a class of chemical compounds that are highly susceptible to a large variety of chemical changes. Among these chemical reactions is one that is of particular value because it gives rise to colored compounds; namely, the ability of diazonium compounds to combine (couple) with imidazoles, phenols, and amines in alkaline solutions. *p*-Phenyldiazonium sulfonate, usually called diazobenzenesulfonic acid, is particularly valuable for colorimetric determinations because it is comparatively stable in water solutions, it does not couple with itself to give a highly colored compound, it is easy to prepare, the dyes formed from it are sufficiently soluble in alkaline solutions to render the use of a colorimeter possible, and the colors produced, usually orange to red, are easily compared because they do not readily produce retinal fatigue. It is not surprising then, to find that *p*-phenyldiazonium sulfonate has been used for some time as a qualitative test for imidazoles, phenols, and amines.[2,3] Up to the present time two methods have been proposed for the use of this diazonium salt in the quantitative estimation of imidazoles;[4,5]

[1] Griess, P., *Ann. chem. Pharm.*, 1866, cxxxvii, 39.

[2] Ehrlich, P., *Z. klin. Med.*, 1882, v, 285; *Char. Ann.*, 1883, viii, 140; *Deutsch. med. Woch.*, 1883, ix, 549; 1884, x, 419.

[3] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

[4] Weisz, M., and Ssobolew, N., *Biochem. Z.*, 1913–14, lviii, 119.

[5] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

but as far as is known to the authors this reagent has not been used, heretofore, for the quantitative estimation of phenols. This paper contains a detailed description of methods for the micro-chemical colorimetric determination of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine.

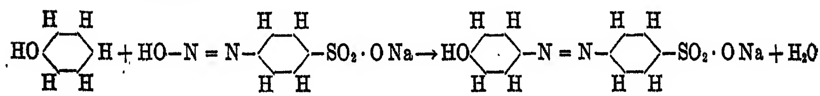
From the standpoint of color development, these phenols can be divided into three classes; namely,

1. Phenols in which the para position is not occupied by a second substituent.

2. Phenols in which the para position is occupied by a second substituent *that does not contain an amino group.*

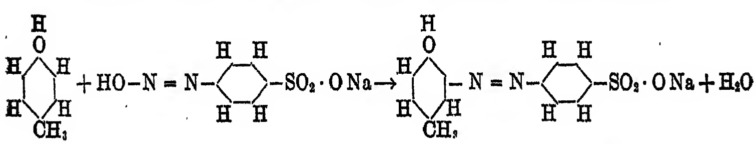
3. Tyrosine and tyramine.

When the position *para* to the phenol radical *is not occupied,* as in phenol, *o*-cresol, and *m*-cresol, the substitution with *p*-phenyldiazonium sulfonate occurs, at least predominantly, in this position.



The coupling is so rapid that about 95 per cent of the color has appeared before a reading can be taken with a Duboscq colorimeter. The color is fully developed in about 2 minutes and it does not change for about 3 to 5 minutes. In these cases the color is predominantly *yellow* with just a dash of red.

When the position para to the phenol radical *is occupied,* as in *p*-cresol and the aromatic hydroxy-acids, the substitution with *p*-phenyldiazonium sulfonate must occur in the ortho position.



The initial rate of coupling in this case, is somewhat slower than that of phenol; but a color of maximum intensity is, nevertheless, obtained in about 3 minutes. The colors produced are predominantly red so that a neutral solution of Congo red can serve as a comparison standard. These colors are stable for from 3 to 5

minutes. The stability of the color and the character of the change that the color undergoes after it has reached its maximum intensity is a fairly accurate index of the composition of the aromatic hydroxy-acid that is present, if only one of these acids is present. This is discussed in detail in the experimental section of the paper.

Tyrosine and tyramine, in small concentrations, show an anomalous behavior toward *p*-phenyldiazonium sulfonate in alkaline solutions. When a solution of tyrosine or tyramine is first added to the alkaline reagent, a pink color begins to develop promptly as in the case of the aromatic hydroxy-acids. After about 30 seconds, however, the color changes sharply to yellow and fades. The intensities of the yellow colors so produced are not directly proportional to the amount of tyrosine or tyramine present. Obviously, then, the usual procedure is worthless for the estimation of either tyrosine or tyramine.

The sharp change in color from pink to yellow suggested a chemical change, perhaps a tautomeric shift. In an effort to stabilize the pink color, or rather to revive it after the initial reaction had gone to completion, we added a small amount of a concentrated solution of sodium hydroxide. The color was intensified and some of the pink tint was restored; but again the color intensities were not directly proportional to the concentration of the phenol. The phenomena noted were so similar to those that one would expect from a compound in which a tautomeric equilibrium existed between a carbonyl form and an enol form that we thought to stabilize the carbonyl derivative, which we believed to be most highly colored, by allowing it to react with hydroxylamine to form an oxime. The addition of hydroxylamine hydrochloride to the alkaline reagent did *not* give rise to an intensification of color; but when sodium hydroxide was added previous to the addition of hydroxylamine, *a very intense bluish red color was produced which was directly proportional to the amount of tyrosine or tyramine added.* Briefly then, the method for estimating either tyrosine or tyramine consists of

1. A reaction between tyrosine or tyramine and *p*-phenyldiazonium sulfonate in an alkaline—sodium carbonate—solution which gives rise to a primary yellow color. This reaction is allowed to proceed for 5 minutes.

2. The addition of 2 cc. of 3.0 N NaOH, after the initial reaction period of 5 minutes, which intensifies the color, stops any further action between the phenol and the diazonium salt and converts the diazonium salt into a sodium diazotate which cannot couple with the hydroxylamine that is to be added later. The alkali is allowed to act for 1 minute.

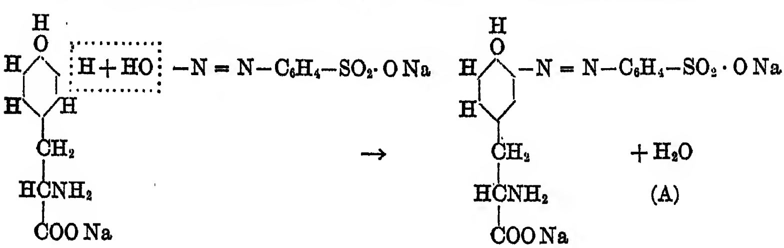
3. The addition of 0.10 cc. of a 20 per cent solution of hydroxylamine hydrochloride, which gives rise to a very intense bluish red color that is stable for at least half an hour.

An explanation of the chemical reactions involved must account for the following facts: Tyrosine and tyramine are the only phenols, that we have examined, that give a color intensification with sodium hydroxide and hydroxylamine. Since oxyphenyllactic acid, which is structurally identical with tyrosine excepting that an OH group replaces the NH_2 group, does not give a color intensification with NaOH and NH_2OH, *the NH_2 group must play a rôle in the color development with these phenols.*

A number of other compounds in which a tautomeric equilibrium exists between a carbonyl form and an enol form, for example acetaldehyde, acetone, and acetoacetic acid, also give an intense color under the conditions just described. The color produced is almost identical with that obtained with tyrosine or tyramine.

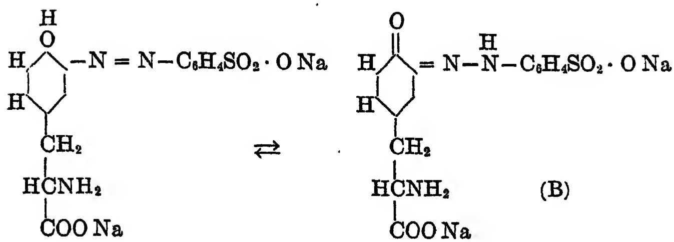
We offer the following explanation tentatively for these color phenomena. Work is now under way to determine the correctness of the following formulations.

Since the position para to the phenol group in tyrosine and tyramine is occupied by an alanyl side chain, the substitution with diazobenzenesulfonic acid must occur in the ortho position.



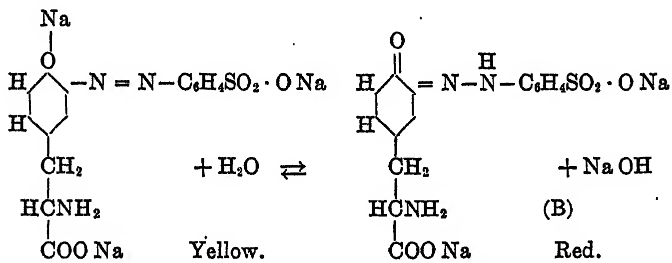
(A)

Compound A—the yellow phenol form—would be in tautomeric equilibrium with a small amount of the supposedly red quinoid form



(B)

In a solution made alkaline with sodium carbonate, practically all of the compound would exist in its phenol form A. The addition of a strong alkali, like sodium hydroxide, would give rise to the sodium phenate derivative of A which, because of its high degree of dissociation, would pass more easily into the quinoid derivative than the phenol itself.

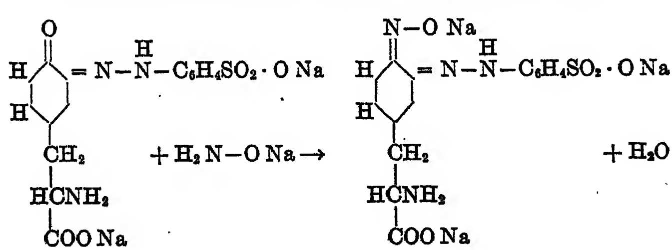


Yellow. (B) Red.

The result would be an intensification of color with the influx of red. The amount of quinoid derivative formed under the influence of alkali must, nevertheless, be *small* because the color intensification is not very great. The addition of hydroxylamine hydrochloride to such a strongly alkaline solution would give rise to the following reactions.

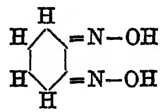
1. $NH_2OH{\cdot}HCl + 2\,NaOH \rightarrow H_2N{-}O\,Na + NaCl + H_2O$

2.



One would expect the quinoneoximehydrazone derivative (C) formed in this way to be dark red. Such *o*-quinoneoximehydrazones seem not to have been studied; but the para derivatives have been known for some time.[6] *They all give deep red alkaline solutions.*

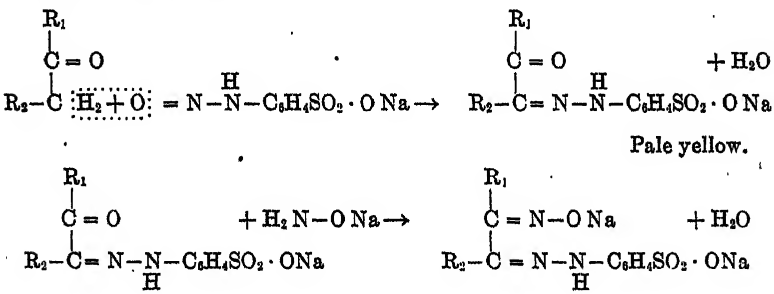
o-Quinonedioxime



which is a closely related compound, also gives a deep red alkaline solution.[7]

Just why tyrosine should give a highly colored quinoneoximehydrazone while *p*-oxyphenyllactic acid does not give such a highly colored derivative, is a problem for future investigation. The rôle played by the amino group in the side chain of tyrosine and tyramine is still obscure.

The reactions that probably occur when certain aldehydes and ketones are treated with diazobenzenesulfonic acid, sodium hydroxide, and hydroxylamine can be represented by the following type equations.



We believe that the red compounds formed with acetaldehyde, acetone, and acetoacetic acid are the sodium salts of hydrazoxime derivatives. Von Pechmann and Wehsarg[8] have prepared a hydrazoxime derivative of methyl glyoxal which is similar to the compound that we believe to be formed with acetone.

[6] Borsche, W., *Ann. chem. Pharm.*, 1907, ccclvii, 171.
[7] Hantzsch, A., and Glover, W. H., *Ber. chem. Ges.*, 1907, xl, 4344.
[8] von Pechmann, H., and Wehsarg, K., *Ber. chem. Ges.*, 1888, xxi, 2996.

$$\begin{array}{l} \mathrm{H} \\ \mathrm{C = N{-}OH} \\ | \\ \mathrm{C = N{-}NH{-}C_6H_5} \\ | \\ \mathrm{CH_3} \end{array}$$

von Pechmann and Wehsarg's hydrazoxime.

$$\begin{array}{l} \mathrm{H\quad\ \ H} \\ \mathrm{C = N{-}N{-}C_6H_4{-}SO_2 \cdot OH} \\ | \\ \mathrm{C = N{-}OH} \\ | \\ \mathrm{CH_3} \end{array}$$

Hydrazoxime formed from acetone.

They give no data on the color of the compound in alkaline solutions although they found the alcoholic solution to be yellow and the sulfuric acid solution to be dark red.

EXPERIMENTAL.

Reagents Employed.

Most of the reagents employed in these estimations are identical with those employed by us in the estimation of imidazoles.[5] A detailed description is repeated here for the sake of completeness.

Stock Sulfanilic Acid.—Sulfanilic acid (4.5 gm.) is mixed with 45 cc. of 37 per cent hydrochloric acid (sp. gr. 1.19) in a 500 cc. volumetric flask. Water is then added to the mark. The solid dissolves slowly; but completely.

Stock Sodium Nitrite.—25 gm. of 90 per cent sodium nitrite are dissolved in water and diluted to 500 cc. in a volumetric flask.

Sodium Carbonate.—Baker and Adamson's anhydrous sodium carbonate (5.50 gm.) is dissolved in water and diluted to exactly 500 cc. We recommend the above grade because we have found it to give uniform results. The purity of this carbonate is a very important factor in the color development. Some grades of carbonate contain impurities that give yellow colors of inferior intensities. The finished carbonate solution must be preserved in a glass vessel that has little tendency to dissolve in alkali. Pyrex glass vessels have proved to be entirely satisfactory.

Stock Methyl Orange.—Vacuum-dried Grübler's methyl orange (0.5000 gm.) is dissolved in water and diluted to exactly 500 cc. This solution keeps indefinitely.

Stock Congo Red.—Vacuum-dried Grübler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volumet-

ric flask. Water is then added to the mark. This solution keeps indefinitely.

Stock Acid Fuchsin.—Vacuum-dried Harmer's acid fuchsin (2.5000 gm.) is dissolved in water and diluted to exactly 500 cc. in a volumetric flask. The thymol-preserved solution keeps indefinitely.

Stock Phenol Red.—Hynson, Westcott and Dunning's phenol red (0.0500 gm.) is dissolved in water and diluted to exactly 500 cc. The thymol-preserved aqueous solution can be kept for at least 1 year. The alcoholic solution employed by many investigators for pH determinations deteriorates quite rapidly and cannot be used in this work.

Standard Indicator Solutions.—For the estimation of phenol, a solution containing 10 cc. of stock phenol red in a total aqueous volume of 100 cc. is employed. Redistilled water should be used for dilutions and the volumetric flask should be thoroughly rinsed with distilled water before the phenol red solution is introduced. It is best to prepare a fresh standard every day. The color so obtained matches that produced by phenol perfectly. When this standard indicator solution has been used for comparisons the symbol (Ph−R) is suffixed to the reading obtained.

For the estimation of o- and m-cresol, a solution containing 5 cc. of stock methyl orange in a total aqueous volume of 500 cc. is employed. The color so obtained matches that produced by *o*- and *m*-cresol almost perfectly. When this standard indicator solution has been used for comparisons the symbol (MO) is suffixed to the reading obtained.

For the estimation of p-cresol, and p-oxyphenylacetic, p-oxyphenylpropionic, and p-oxyphenyllactic acids, a solution containing 1.00 cc. of stock Congo red in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (CR) is suffixed to the reading obtained.

For the estimation of tyrosine or tyramine, a solution containing 1 cc. of stock acid fuchsin and 1.8 cc. of stock methyl orange in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (F−MO) is suffixed to the reading obtained.

Preparation of p-Diazobenzenesulfonic Acid Solution (The Reagent).

1.50 cc. each of the stock sulfanilic acid and sodium nitrite solutions are measured into a 50 cc. volumetric flask. The flask is then immersed in an ice bath for 5 minutes. Then 6.00 cc. more of the stock sodium nitrite solution are added and the well mixed solution is again allowed to lie in the ice bath for 5 minutes. Distilled water is then added to the mark and the flask returned to the ice bath where it is kept. This reagent must not be used for at least 15 minutes after diluting with water. We have found it to give perfect results after 24 hours. It is best, however, to prepare a fresh reagent every day.

Preparation of the Phenols.

Phenol.—Merck's highest purity phenol was distilled *in vacuo.* The product collected boiled at 95° in a vacuum of 10 mm. of Hg. The colorless oil solidified readily. The colorless crystals so obtained had a melting point of 43°. The substance was assumed to be 100 per cent pure phenol.

o-Cresol.—A chemically pure product obtained from a local supply house was doubly distilled *in vacuo.* The colorless oil obtained boiled at 81° at a pressure of 10 mm. and at 188° under atmospheric pressure. The oil solidified on standing. The colorless crystals so obtained had a melting point of 32°. It was assumed to be 100 per cent pure *o*-cresol.

m-Cresol.—A chemically pure product obtained from a local supply house was doubly distilled *in vacuo.* The colorless oil so obtained boiled at 104° under 10 mm. pressure and at 201° under atmospheric pressure. It did not solidify. It was assumed to be 100 per cent pure *m*-cresol.

p-Cresol.—The *p*-cresol was obtained as a by-product in the preparation of tyramine.[9] The substance boiled at 90–91° at a pressure of 10 mm. and at 198° under atmospheric pressure. The oil solidified after standing for some time. The colorless crystals so obtained melted at 36°. It was assumed to be 100 per cent pure *p*-cresol.

[9] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 585.

Oxyphenylacetic Acid.—This substance was obtained as a by-product in the preparation of tyramine.[9] The colorless solid had the following properties.

1. It melted at 150°.
2. It left no residue on ignition.
3. The solid (0.1000 gm.) was dissolved in 10 cc. of water and titrated with 0.1 N NaOH using phenolphthalein as indicator. Exactly 6.59 cc. of the alkali were required to produce the first change in the indicator. The amount demanded by the formula is 6.58 cc. The substance was, therefore, 100 per cent pure.

Oxyphenylpropionic Acid.—This compound was prepared synthetically as follows.

Cinnamic acid was reduced, with sodium amalgam, to hydrocinnamic acid.[10]

p-Nitrophenylpropionic acid was then prepared by a modification of the method of Beilstein and Kuhlberg.[11]

Stöhr[12] prepared *p*-aminophenylpropionic acid by reducing the ethyl ester of *p*-nitrophenylpropionic acid with zinc and hydrochloric acid. He isolated the zinc double salt of the acid and treated this with acid and sodium nitrite to prepare *p*-oxyphenylpropionic acid. We reduced *p*-nitrophenylpropionic acid (6 gm.) directly with zinc (14 gm.) and hydrochloric acid (50 cc. of the 37 per cent acid) in alcoholic solution (70 cc. of 95 per cent alcohol) adding the acid slowly and keeping the temperature below 30°. The colorless liquid obtained, after the reaction had been allowed to proceed for 24 hours, was treated with 23.5 cc. of 95 per cent sulfuric acid and subjected to a distillation *in vacuo* at 60°. The pale yellow solid so obtained was dissolved in water (200 cc.) treated with sulfuric acid (20 cc. of the 95 per cent acid) and cooled in an ice bath. Sodium nitrite (60 cc. of a 5 per cent solution) was poured into the cold liquid. The resulting solution was allowed to remain in the ice bath for 1 hour after which it was treated with 250 cc. of water and heated to the boiling point. There was a copious evolution of nitrogen; but practically no tar formation. The aqueous solution was

[10] Fischer, E., Anleitung zur Darstellung organischer Präparate, Brunswick, 8th edition, 1908, 39.

[11] Beilstein, F., and Kuhlberg, A., *Ann. chem. Pharm.*, 1872, clxiii, 132.

[12] Stöhr, C., *Ann. chem. Pharm.*, 1884, ccxxv, 60.

cooled and extracted with ether. Removal of the ether, by distillation, left a pale yellow crystal cake weighing 4.5 gm. which was recrystallized first from water and then from hot benzene. The colorless solid finally obtained had the following properties.

1. It melted sharply at 130° (corrected).
2. It left no residue on ignition.
3. 0.166 gm. of the vacuum-dried solid was dissolved in water and the solution titrated with 0.20 N NaOH using phenolphthalein as indicator. Exactly 5.00 cc. of alkali were required. This is the amount demanded by the formula. The solid was 100 per cent pure.

p-Oxyphenyllactic Acid.—This was prepared from tyrosine by the method of Kotake.[13] The colorless solid obtained after drying to constant weight at 105° had the following properties.

1. It melted sharply at 172° (corrected).
2. It left no residue on ignition.
3. 0.25 gm. was dissolved in water and the solution titrated with 0.1 N NaOH using phenolphthalein as indicator. Exactly 13.70 cc. of alkali were required, which is almost exactly the amount (13.74 cc.) required by the formula. The solid was 100 per cent pure.

Tyrosine.—The tyrosine used in this work was prepared from horn. The pure white, asbestos-like solid had the following properties.

1. It did not melt at a temperature of 290°C.
2. It was free from ammonia.
3. It left no residue on ignition.
4. It contained no cystine.
5. The ammonia obtained from 0.1000 gm. of the solid—Kjeldahl method—neutralized 5.50 cc. of 0.10 N HCl as compared with 5.52 cc. required by the formula. The substance was 100 per cent pure.

Tyramine Hydrochloride.—The synthetic preparation and properties of this compound have been previously described by us.[9] The product used was 98.14 per cent pure tyramine hydrochloride. The impurity consisted entirely of sodium chloride.

[13] Kotake, Y., *Z. physiol. Chem.*, 1910, lxv, 398.

Procedure for Estimating Phenols Other than Tyrosine or Tyramine.

Process I.

The method used in developing the tables as well as the general procedure for estimating phenols other than tyrosine and tyramine is illustrated by the following example.

(1–X) cc. of water and 5 cc. of the 1.1 per cent sodium carbonate solution are accurately measured into the right-hand cylinder of a Duboscq colorimeter. 2 cc. of reagent are measured into a 5 second delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder are then thoroughly mixed by allowing the liquid to flow repeatedly up the inclined tube as far as safety from loss will permit. The mixing should not take over 30 seconds. X cc. of the phenol solution is allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder are mixed *thoroughly* as above.[14] The test cylinder is then transferred to the colorimeter and set at 20 mm. The left-hand cylinder, which should contain the appropriate standard indicator solution is then adjusted constantly until a maximum reading has been obtained.

The most accurate readings can be obtained by choosing such an amount of phenol solution that the standard indicator-containing cylinder has to be set at from 5 to 20 mm.

The method described here can be used on quantities of the phenol-containing solution varying from 0.01 to 1 cc. The combined volume of water and phenol solution used should always be 1 cc. Thus, if 0.10 cc. of the phenol solution is to be used, 0.90 cc. of water is added to the test cylinder. Then X equals 0.10 cc. and 1–X equals 0.90 cc.

Estimation of Small Amounts of Phenol.

A stock 1.00 per cent phenol solution was prepared by dissolving 2.0000 gm. of the pure solid in water and diluting to exactly 200 cc. From this the standard solution was prepared by dilut-

[14] Process II described on page 257 is the same as Process I up to this point.

ing 1 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of phenol.

The color produced with phenol is very intense. It develops so rapidly that it has reached 95 per cent of its maximum value before a reading can be taken. A maximum color is obtained within 2 minutes of the time that the phenol is added to the alkaline reagent. The color remains of maximum intensity for about 5 minutes, then fades slowly without changing appreciably in tint. Most people find this greenish yellow color hard to compare, without practice, because of the speed with which it produces retinal fatigue. Accurate readings can only be obtained by observing the colors briefly, setting the cylinder rapidly and making the fine adjustment after a brief interval of rest. When this is done the readings can be checked with an accuracy of about 1 per cent.

The previously described (Ph–R) standard indicator solution was used for comparisons in the compilation of Table I. This table shows clearly that the color production is directly proportional to the amount of phenol used.

Where only a few determinations are to be carried out it is usually simpler to compare the color produced by an unknown with that produced by a measured amount of a standard phenol solution, the two colors being prepared simultaneously. When the amount of standard phenol used is nearly equal to that in the test liquid, very accurate results can be obtained by this method.

Estimation of Small Amounts of o-Cresol.

A stock 0.1 per cent *o*-cresol solution was prepared by dissolving 1.0000 gm. of the pure solid in water and diluting to exactly 1,000 cc. From this the standard solution was prepared by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *o*-cresol.

The color produced with *o*-cresol is very intense. Its speed of development is like that of phenol. The color, which is predominantly yellow but which contains slightly more pink than that produced by phenol, remains of maximum intensity and permanent tint for about 3 minutes; then it fades rapidly and acquires a redder tint. This color is easier to compare than that of phenol.

TABLE I.

Estimation of Small Amounts of Phenol.

Depth of indicator solution (Ph—R) required to match the color in the test cylinder.	Phenol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.3	0.000002
3.5	0.000003
4.6	0.000004
5.8	0.000005
6.9	0.000006
8.1	0.000007
9.2	0.000008
10.4	0.000009
11.5	0.000010
12.7	0.000011
13.8	0.000012
15.0	0.000013
16.1	0.000014
17.3	0.000015
18.4	0.000016
19.6	0.000017
20.7	0.000018
21.9	0.000019
23.0	0.000020
24.2	0.000021
25.3	0.000022
26.5	0.000023
27.6	0.000024
28.8	0.000025
29.9	0.000026
31.1	0.000027
32.2	0.000028
33.4	0.000029
34.5	0.000030

The previously described (MO) standard indicator solution was used for comparisons in the compilation of Table II. This table shows clearly that the color produced is directly proportional to the amount of *o*-cresol used.

TABLE II.

Estimation of Small Amounts of o-Cresol.

Depth of indicator solution (MO) required to match the color in the test cylinder.	*o*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.4	0.000002
3.6	0.000003
4.8	0.000004
6.0	0.000005
7.3	0.000006
8.5	0.000007
9.7	0.000008
10.9	0.000009
12.1	0.000010
13.3	0.000011
14.5	0.000012
15.7	0.000013
16.9	0.000014
18.1	0.000015
19.4	0.000016
20.6	0.000017
21.8	0.000018
23.0	0.000019
24.2	0.000020
25.4	0.000021
26.6	0.000022
27.8	0.000023
29.0	0.000024
30.2	0.000025
31.5	0.000026
32.7	0.000027
33.9	0.000028
35.1	0.000029
36.3	0.000030

Estimation of Small Amounts of m-Cresol.

A stock 0.10 per cent solution of *m*-cresol was prepared by dissolving 1.000 gm. of the colorless oil in water and diluting to exactly 1,000 cc. From this the standard solution was prepared

by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *m*-cresol.

TABLE III.

Estimation of Small Amounts of m-Cresol.

Depth of indicator solution (MO) required to match the color in the test cylinder.	*m*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.0	0.000001
1.9	0.000002
2.9	0.000003
3.9	0.000004
4.9	0.000005
5.8	0.000006
6.8	0.000007
7.8	0.000008
8.7	0.000009
9.7	0.000010
10.7	0.000011
11.6	0.000012
12.6	0.000013
13.6	0.000014
14.6	0.000015
15.5	0.000016
16.5	0.000017
17.5	0.000018
18.4	0.000019
19.4	0.000020
20.4	0.000021
21.3	0.000022
22.3	0.000023
23.3	0.000024
24.3	0.000025
25.2	0.000026
26.2	0.000027
27.2	0.000028
28.1	0.000029
29.1	0.000030

The intense color produced with *m*-cresol is qualitatively identical with that obtained with *o*-cresol. In this case, however,

the time of complete development is about 5 minutes although most of the color appears immediately. At first the color is slightly more yellow than the (MO) comparison standard. In the course of 3 minutes the match is perfect and a maximum of color is obtained in 5 minutes. The color fades slowly and becomes pinker. The previously described (MO) standard indicator solution was used for comparisons in the compilation of Table III. This table shows clearly that the color production is directly proportional to the amount of *m*-cresol used.

Estimation of Small Amounts of p-Cresol.

A stock 0.10 per cent solution of *p*-cresol was prepared by dissolving 1.000 gm. of the colorless solid in water and diluting to exactly 1,000 cc. From this the standard solution was prepared by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-cresol.

In this case the color, which is predominantly red, develops to its maximum intensity during the 30 seconds required to mix the contents of the test cylinder. The readings recorded are those obtained *immediately* after the test cylinder was transferred to the colorimeter. The comparison must be made within 2 minutes during which time the color remains of constant and maximum intensity. Then the test liquid, although it changes but little in intensity, rapidly acquires a cloudy appearance which renders further comparisons untrustworthy. This same cloudy appearance is also obtained when the test liquid contains a finely divided precipitate which leads us to believe that an insoluble compound appears, after 2 minutes, although none can be seen with the naked eye.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table IV. As can be seen from the table, the color production is directly proportional to the amount of *p*-cresol used.

Estimation of Small Amounts of p-Oxyphenylacetic Acid.

A stock 1.00 per cent solution of *p*-oxyphenylacetic acid was prepared by dissolving 1.0000 gm. of the colorless solid in water and diluting to 100 cc. This solution was preserved with chloroform.

From it, the standard solution was prepared by diluting 1 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenylacetic acid.

TABLE IV.

Estimation of Small Amounts of p-Cresol.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
2.5	0.000005
3.0	0.000006
3.5	0.000007
4.0	0.000008
4.5	0.000009
5.0	0.000010
5.5	0.000011
6.0	0.000012
6.5	0.000013
7.0	0.000014
7.5	0.000015
8.0	0.000016
8.5	0.000017
9.0	0.000018
9.5	0.000019
10.0	0.000020
10.5	0.000021
11.0	0.000022
11.5	0.000023
12.0	0.000024
12.5	0.000025
13.0	0.000026
13.5	0.000027
14.0	0.000028
14.5	0.000029
15.0	0.000030

In this case the color, which is predominantly red, develops to its maximum intensity in about 2 minutes. This color of maximum intensity is permanent for about 5 minutes after which it fades slowly and becomes yellow.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table V. It has a color that is slightly more red than that produced by *p*-oxyphenylacetic acid; but the match is so close that accurate comparisons are easily made. As can be seen from Table V the color production is directly proportional to the amount of *p*-oxyphenylacetic acid used.

TABLE V.

Estimation of Small Amounts of p-Oxyphenylacetic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenylacetic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
3.6	0.000010
4.0	0.000011
4.3	0.000012
4.7	0.000013
5.0	0.000014
5.4	0.000015
5.7	0.000016
6.1	0.000017
6.5	0.000018
6.8	0.000019
7.2	0.000020
7.6	0.000021
7.9	0.000022
8.3	0.000023
8.6	0.000024
9.0	0.000025
9.4	0.000026
9.7	0.000027
10.1	0.000028
10.4	0.000029
10.8	0.000030

Estimation of Small Amounts of p-Oxyphenylpropionic Acid.

A stock 1.00 per cent solution of *p*-oxyphenylpropionic acid was prepared by dissolving 1.0000 gm. of the solid in 30 cc. of 0.20 N NaOH and diluting, with water, to exactly 100 cc. Chloroform was added as a preservative. The standard solution was prepared from the stock solution by diluting 1.00 cc. of the latter

to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenylpropionic acid. The color, which is predominantly red, develops to its maximum intensity in about 2 minutes and undergoes no change for from 1 to 3 minutes depending

TABLE VI.

Estimation of Small Amounts of p-Oxyphenylpropionic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenylpropionic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
3.1	0.000010
3.4	0.000011
3.7	0 000012
4.0	0.000013
4.3	0.000014
4.6	0.000015
5.0	0.000016
5.3	0.000017
5.6	0.000018
5.9	0.000019
6.2	0.000020
6.5	0.000021
6.8	0.000022
7.1	0.000023
7.4	0.000024
7.7	0.000025
8.1	0.000026
8.4	0.000027
8.7	0.000028
9.0	0.000029
9.3	0.000030
9.6	0.000031
9.9	0.000032
10.2	0.000033
10.5	0.000034
10.8	0.000035
11.1	0.000036
11.5	0 000037
11.8	0.000038
12.1	0.000039
12.4	0.000040

upon the quantity of *p*-oxyphenylpropionic acid present and the room temperature. This period of color constancy is followed, rather sharply, by a rapid decline in color intensity, a cloudy appearance and a change in tint. The values recorded in Table VI are those obtained just previous to this change.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table VI. This table shows that the color production is directly proportional to the amount of *p*-oxyphenylpropionic acid present.

Estimation of Small Amounts of p-Oxyphenyllactic Acid.

A stock 1.00 per cent solution of *p*-oxyphenyllactic acid was prepared by dissolving 1.0000 gm. of the pure solid in 56 cc. of 0.1 N NaOH and diluting with water to exactly 100 cc. Chloroform was added as a preservative. The standard solution was prepared by diluting 1.00 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenyllactic acid.

The color, which is predominantly red, develops to its maximum intensity in about 4 minutes and undergoes no change for about 5 minutes. The (CR) standard, which was used for comparisons in the compilation of Table VII, is slightly more red than the color produced by *p*-oxyphenyllactic acid; but the discrepancy in tint is too slight to interfere with the accuracy of the comparisons. The slowness of the color development, the slight yellow tint, and the stability of the color are characteristics for oxyphenyllactic acid. In this case there is no sharp color change and the liquid does not become cloudy as in the case of oxyphenylpropionic acid.

The tabular values have been carried out to the second place because these figures are obviously more correct than the actual readings which can, of course, be obtained with an accuracy of only one decimal place. Thus for 0.000019 gm. of *p*-oxyphenyllactic acid a reading of either 4.70 or 4.80 mm. is obtained; but the correct reading would be 4.75. This figure (4.75) therefore, appears in the table. As in the case of the other phenols, the color production is directly proportional to the amount of the phenol used.

TABLE VII.

Estimation of Small Amounts of p-Oxyphenyllactic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	p-Oxyphenyllactic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
2.5	0.000010
2.75	0.000011
3.0	0.000012
3.25	0.000013
3.5	0.000014
3.75	0.000015
4.0	0.000016
4.25	0.000017
4.5	0.000018
4.75	0.000019
5.0	0.000020
5.25	0.000021
5.5	0.000022
5.75	0.000023
6.0	0.000024
6.25	0.000025
6.5	0.000026
6.75	0.000027
7.0	0.000028
7.25	0.000029
7.5	0.000030
7.75	0.000031
8.0	0.000032
8.25	0.000033
8.5	0.000034
8.75	0.000035
9.0	0.000036
9.25	0.000037
9.5	0.000038
9.75	0.000039
10.0	0.000040
10.25	0.000041
10.5	0.000042
10.75	0.000043
11.0	0.000044
11.25	0.000045
11.5	0.000046
11.75	0.000047
12.0	0.000048
12.25	0.000049
12.5	0.000050

Procedure for the Estimation of Small Amounts of Tyrosine and Tyramine.

Process II.

As has already been pointed out in the introduction, tyrosine and tyramine show an anomalous behavior toward *p*-phenyldiazonium sulfonate in alkaline—sodium carbonate—solutions. When a solution of tyrosine or tyramine is added to the alkaline reagent, a pink color develops promptly, as in the case of the aromatic hydroxy-acids. After about 30 seconds, however, the color changes sharply to yellow and fades. The pink color is too evanescent to compare and the intensities of the yellow colors are not directly proportional to the amount of tyrosine or tyramine used. The procedure that has been found to give satisfactory results with the other phenols, and with imidazoles, cannot be used for the estimation of tyrosine or tyramine. The procedure finally adopted is given below; the reasons for its adoption and an explanation of the chemical reactions involved have already been given in the introduction.

Process I described for the other phenols (see page 246) is followed as far as foot-note 14. The test cylinder is allowed to stand for exactly 5½ minutes from the time that the tyrosine or tyramine solution began to mix with the alkaline reagent. This gives rise to a primary color that is yellow to orange and whose intensity is not directly proportional to the amount of the phenol used.

Sodium hydroxide (2.00 cc. of a 3.0 N solution) is added and the contents of the cylinder thoroughly mixed as before. This gives rise to a marked color intensification with a change of tint toward red, the color still being, however, predominantly yellow.

Exactly 1 minute after the sodium hydroxide solution began to mix with the liquid in the test cylinder, 0.10 cc. of a 20 per cent solution of hydroxylamine hydrochloride[15] is rapidly introduced and the contents of the cylinder are again thoroughly and rapidly mixed. At first there is no change in color. Then suddenly, after a latent period of from 5 to 10 seconds, an intense bluish red color

[15] This was obtained from the Special Chemicals Co., Highland Park, Illinois.

develops. This secondary color develops to its full intensity while the cylinder and contents are being agitated (30 seconds) and changes very little in tint or intensity in half an hour. It is best to introduce the hydroxylamine solution from a rapid delivery 0.10 cc. pipette and to have the liquid well mixed before the secondary color *begins* to develop. The test cylinder is transferred to the right-hand side of the Duboscq colorimeter and set at 25 mm. The left-hand cylinder, which contains the previously described (F–MO) comparison standard, is then adjusted until the two halves of the field are identical in tint and intensity.

The color is easily compared and the determinations are accurate to about 0.5 to 1.5 per cent. If the process is properly carried out, the correction blank (see later) is about 0.30 mm. (F–MO). This amount must be subtracted from the actual reading to obtain the values recorded in Table VIII. We have found that a high laboratory temperature raises this correction blank from 0.10 to 0.20 mm. *The hydroxylamine solution must not be introduced until the sodium hydroxide has been allowed to react with the alkaline reagent for at least 1 minute;* otherwise a colored compound is produced with the hydroxylamine.

The tabular values are accurate for laboratory temperatures ranging from 18 to 25°. At lower temperatures the colors are very slightly less intense and at higher temperatures they are slightly more intense. It is always advisable, before carrying out a determination on an unknown, to run a few determinations on a standard tyrosine solution to be certain that the tabular values are accurate for the existing laboratory conditions.

It is a curious coincidence that the tabular values are identical for both tyrosine and tyramine hydrochloride. The same table may, therefore, be used in the estimation of either.

A stock 1.00 per cent solution of tyrosine was prepared by dissolving 2.0000 gm. of the pure solid in 75 cc. of 1.0 N HCl and diluting with water to 200 cc. From this the standard solution was prepared by diluting 1 cc. to 100 cc. in a volumetric flask. Each cc. of the standard solution contained 0.0001 gm. of tyrosine.

A stock 1.00 per cent solution of tyramine hydrochloride was prepared by dissolving 2.0379 gm. of the 98.14 per cent solid in water and diluting to exactly 200 cc. Chloroform was added as a preservative. The standard solution was prepared by diluting

1.00 cc. of the stock solution to 100 cc. in a volumetric flask. Each cc. of the standard solution contained 0.0001 gm. of tyramine hydrochloride.

TABLE VIII.

Estimation of Small Amounts of Tyrosine and Tyramine Hydrochloride.

Depth of indicator solution (F—MO) required to match the color in the test cylinder.	Tyrosine or tyramine hydrochloride in the test cylinder (total volume 10.1 cc.) test cylinder set at 25 mm.
mm.	*gm.*
4.0	0.000005
4.8	0.000006
5.6	0.000007
6.4	0.000008
7.2	0.000009
8.0	0.000010
8.8	0.000011
9.6	0.000012
10.4	0.000013
11.2	0.000014
12.0	0.000015
12.8	0.000016
13.6	0.000017
14.4	0.000018
15.2	0.000019
16.0	0.000020
16.8	0.000021
17.6	0.000022
18.4	0.000023
19.2	0.000024
20.0	0.000025
20.8	0.000026
21.6	0.000027
22.4	0.000028
23.2	0.000029
24.0	0.000030

The Correction Blank.

When the reagent and alkali are mixed in the absence of a phenol derivative, a very pale yellow color is produced in the course of 5 minutes. This color has an intensity value equivalent to about 0.30 mm. of any of the standard indicator solutions used. This

same amount of color is also produced in the presence of phenol derivatives along with the color produced by the phenol; so the readings obtained are high by about 0.30 mm. in every case. It is necessary, therefore, to subtract 0.30 mm. from the readings obtained before comparison with the tables.

Substances That Do and Do Not Interfere with the Quantitative Determination of Phenols.

The tests for interference were carried out as follows, unless otherwise specified.

Tyrosine (1.00 cc. of the stock 1 per cent solution) was mixed, in a 100 cc. graduated precision cylinder, with quantities of the interfering substances as given below. Water was added to give a total volume of 100 cc. Then 0.10 and 0.20 cc. portions of this liquid were taken for the colorimetric determinations.

Interference with the determination of tyrosine was studied particularly because this determination involves a heretofore unstudied type of reaction. Phenols other than tyrosine and tyramine are determined by a process identical with that used for imidazoles and the interference with that process has already been studied and reported.

Sodium and potassium chloride, sulfate, phosphate, acetate, and citrate in 5 per cent concentrations do not interfere with the colorimetric determination of tyrosine or tyramine.

Ammonium salts interfere very seriously with the determination. When the concentration of ammonium chloride is 5 per cent, so much of a greenish yellow color is produced that the *presence* of tyrosine would not be suspected. Needless to say, tyrosine could not be determined under these conditions. When the concentration of ammonium chloride is 1 per cent, the interference is still considerable because 0.10 cc. of the solution had a color value equal to 9.0 mm. (F–MO) as compared to a normal value of 8.0 mm.; which is a positive error of 12.5 per cent. The color was quite yellow. When the concentration of ammonium chloride is reduced to 0.5 per cent results were obtained that are fairly satisfactory for now 0.10 cc. had a color value equal to 8.2 mm. (F–MO) which is 102.5 per cent of the correct reading. The color was exactly like that of the standard. *Ammonium salts must not be present in concentrations exceeding 0.5 per cent in liquids that are to be examined for tyrosine colorimetrically.*

Amino-acids also interfere seriously with the colorimetric estimation of tyrosine as can be seen from the following data.

Leucine.—A solution was prepared containing 5 cc. of a 1 per cent leucine solution, 0.20 cc. of a 1 per cent tyrosine solution, and sufficient water to give a total volume of 10 cc. Of this solution

0.05 cc. had a color value equivalent to 10.6 mm. (F – MO) and
0.10 " " " " " " 18.7 " (F – MO).

The color contained far more yellow than the comparison standard. Both of these values are high, the 0.05 cc. reading being 30 per cent high and the 0.10 cc. reading being 15 per cent high. This excess of color is due to a yellow compound that is formed when leucine reacts with *p*-phenyldiazonium sulfonate. In this case the ratio of leucine to tyrosine was 25 to 1.

A second liquid was prepared containing 1.00 cc. of a 1 per cent leucine solution, 0.10 cc. of a 1 per cent tyrosine solution, and sufficient water to give a total volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.4 mm. (F – MO) and
0.20 " " " " " " 16.8 " (F – MO).

The color obtained contained slightly more yellow than the comparison standard; but the match was very good. These values are 5 per cent higher than they should be. The ratio of leucine to tyrosine in this case was 10 to 1.

These experiments show that *leucine interferes seriously with the colorimetic estimation of tyrosine when the ratio of leucine to tyrosine exceeds 10 to 1.*

Glycine.—A solution was prepared containing 0.30 cc. of a 1 per cent glycine solution and 0.10 cc. of a 1 per cent tyrosine solution in a total aqueous volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F – MO) and
0.20 " " " " " " 16.0 " (F – MO).

The color produced matched that of the standard perfectly. The values obtained are exactly what they would have been if glycine had not been present. When the ratio of glycine to tyrosine is 3 to 1, the colorimetric estimation of tyrosine is not interfered with.

A second solution was prepared containing 0.50 cc. of a 1 per cent glycine solution and 0.10 cc. of a 1 per cent tyrosine solution in a total aqueous volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.5 mm. (F − MO) and
0.20 " " " " " " 17.0 " (F − MO).

The color produced was distinctly yellow and not easy to compare with the (F–MO) comparison standard. The values are too high by 6 per cent. Even at this ratio (5 to 1), glycine interferes seriously with the colorimetric determination of tyrosine. With higher concentrations of glycine the interference becomes so pronounced that a determination of any kind is impossible. The color produced is predominantly yellow so that the color due to tyrosine is masked almost completely.

These experiments show that *glycine interferes seriously with the colorimetric estimation of tyrosine when the ratio of glycine to tyrosine exceeds 5 to 1.* From these experiments it is clear that the direct determination of tyrosine colorimetrically in the phosphotungstate filtrate fraction of a protein is impossible by means of this method *as it now stands.* Tyrosine, excepting for that part which can be separated by crystallization, is always associated with a high percentage of other amino-acids. To estimate tyrosine under these conditions we have either to separate tyrosine, or some derivative into which it can be quantitatively converted, from the bulk of the other amino-acids, or to remove the interfering NH_2 groups of these other amino-acids without destroying the colorimetric properties of tyrosine. Attempts are now being made in this laboratory to modify this method so that it will be applicable to the estimation of tyrosine in proteins.

Hydrogen Peroxide.—A solution was prepared containing 0.01 gm. of tyrosine and 0.20 cc. of a 3 per cent commercial hydrogen peroxide solution in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 10.0 mm. (F − MO)

as compared to a normal value of 16.0 mm. The color contained far more yellow than the comparison standard. Hydrogen peroxide is, therefore, a very potent interfering substance.

In its presence extremely low and entirely unreliable values are obtained. Fortunately, hydrogen peroxide can be readily removed from an aqueous solution by treating it with platinized asbestos. An example of such a treatment is given below under formaldehyde.

Formaldehyde.—A liquid was prepared containing 0.01 gm. of tyrosine and 5 cc. of commercial formaldehyde in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 9.2 mm. (F − MO).

The reading should have been 16.0 mm. The color matched that of the comparison standard very well.

A liquid was now prepared containing 0.01 gm. of tyrosine and 2 cc. of formaldehyde in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 14.4 mm. (F − MO).

In this case the reading was low by 10 per cent.

Finally a solution was prepared containing 0.01 gm. of tyrosine and 1.00 cc. of formaldehyde in a total aqueous volume of 100 cc. of which

0.20 cc. had a color value equivalent to 16.0 mm. (F − MO).

This is the normal reading. In this concentration (1.0 per cent) formaldehyde *does not* interfere with the colorimetric estimation of tyrosine.

It is of interest to note that *low* values are obtained when formaldehyde is present. Formaldehyde does *not* give a colored substance with *p*-phenyldiazonium sulfonate. In this respect this aldehyde is very different from acetaldehyde, acetone, and acetoacetic acid since the latter substances give intensely colored compounds with *p*-phenyldiazonium sulfonate in alkaline solution after treatment with sodium hydroxide and hydroxylamine. *Formaldehyde cannot form an olefine enol,* it cannot give rise to hydrazoxime derivatives; the other three carbonyls can. This may account for their different behaviors.

Since it is conceivable that tyrosine determinations might have to be carried out on liquids containing formaldehyde, we sought

for a method that would destroy formaldehyde without injuring tyrosine. Blank and Finkenbeiner[16] have described a method for the quantitative conversion of formaldehyde into sodium formate by means of hydrogen peroxide. We found that this method destroyed formaldehyde and did not destroy tyrosine; but the hydrogen peroxide left after the reaction had gone to completion made it impossible to determine the tyrosine colorimetrically. We then removed the hydrogen peroxide by means of platinized asbestos and found that the concentration of tyrosine had not changed. The experiment was conducted as follows.

A solution was prepared containing 0.01 gm. of tyrosine, 7 cc. of water, 3 cc. of commercial formaldehyde, and 17 cc. of 3 N NaOH. The substances were mixed in a 100 cc. graduated cylinder. After a reaction period of about 15 minutes, hydrogen peroxide (50 cc. of a 3 per cent commercial preparation) was slowly added to the contents of the cylinder *without cooling*. After the evolution of gas had ceased (15 minutes) 0.30 gm. of a 5 per cent platinized asbestos was added to the liquid. The evolution of oxygen was practically nil after 30 minutes. The liquid was neutralized with 5 N sulfuric acid (which took 3 cc.) and diluted to 100 cc. Of the clear colorless liquid obtained after filtration,

0.10 cc. had a color value equivalent to 8.0 mm. (F – MO) and
0.20 " " " " " " "15.9 " (F – MO)

which is equivalent to 0.01 gm. of tyrosine for the entire test liquid, 100 per cent of the starting material. Formaldehyde and hydrogen peroxide do not destroy tyrosine under the conditions of the above experiment. Both of these interfering substances can be completely removed without interfering with the color-producing properties of tyrosine.

Carbonyl-Enol Interference (Acetaldehyde, Acetone, and Acetoacetic Acid).

These three substances have been classified as carbonyl-enols because they are capable of existing in two tautomeric forms; namely,

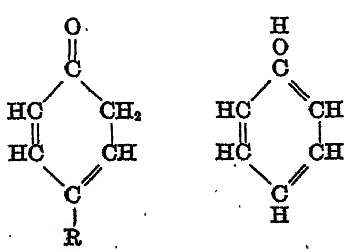
[16] Blank, O., and Finkenbeiner, H., *Ber. chem. Ges.*, 1898–99, xxxi, 2979.

Carbonyl form.	Enol form.
$\begin{array}{c} CH_3-\overset{}{C}{:}O \\ \;\;\;\;\,| \quad\;\; \\ \;\;\;\;\,H \quad\;\; \end{array}$	$\begin{array}{c} H_2C=C-OH \\ \;\;\;\;\;\;\;| \quad\;\;\; \\ \;\;\;\;\;\;\;H \quad\;\;\; \end{array}$
$\begin{array}{c} CH_3-C-CH_3 \\ \Vert \\ O \end{array}$	$\begin{array}{c} CH_3-C=CH_2 \\ \;\;\;\;\;| \quad\;\;\; \\ \;\;\;\;\;OH \quad\;\; \end{array}$
$CH_3-CO-CH_2-COOH$	$\begin{array}{c} \quad\quad\quad\; H \quad\quad\;\; \\ \quad\quad\quad\; | \quad\quad\;\; \\ CH_3-C=C-COOH \\ \;\;\;\;\;\;| \quad\quad\quad\quad\; \\ \;\;\;\;\;\;OH \quad\quad\quad\;\; \end{array}$

These compounds are particularly potent as interfering agents because they produce a color, under the conditions employed in the estimation of tyrosine, that closely resembles that produced by tyrosine. These colors are so intense that as little as 0.00005 gm. of any of the three substances gives sufficient color to be easily read in a colorimeter.

When the carbonyl derivative is first added to the alkaline reagent a brilliant red color is produced that fades in a few seconds and becomes yellow. The addition of sodium hydroxide, after the initial 5 minute reaction period, enhances this color and changes its tint to bluish red. The subsequent addition of hydroxylamine gives rise to an intense bluish red color that is almost identical with that obtained with tyrosine.

The fact that so simple a substance as acetaldehyde should give a color similar to that obtained with tyrosine would lead one to suspect that this carbonyl-enol tautomerism played a rôle in the color production by tyrosine and tyramine (see introduction for the chemical reactions involved). Phenols can also occur in two tautomeric forms that would properly be called carbonyl and enol, respectively



Acetone is the only member of this group upon which we have carried out any extended experiments. A stock 5 per cent solution of acetone was prepared by diluting 5 gm. of the pure product with water to 100 cc. A standard solution was then prepared by diluting 4 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.002 gm. of acetone. When 0.20 cc. of this standard solution (equivalent to 0.0004 gm. of acetone) was mixed with the alkaline reagent and subsequently treated with sodium hydroxide and hydroxylamine as in the tyrosine determination, a color was produced that was equivalent to about 8.5 mm. (F–MO), the color being slightly redder than that of the comparison standard. It was almost impossible to obtain exactly this value on repetition because the slightest change in the primary reaction time produced a large change in the reading finally obtained. We, therefore, tried several experiments (using 0.20 cc. of the standard acetone solution) in which this reaction time was elongated. With a reaction time of 15 minutes a color value of 29.6 mm. (F–MO) was obtained. With a reaction time of 25 minutes a color value of 31.6 mm. (F–MO) was obtained. Obviously then, the 5 minute reaction period that we found to be ample for the complete development of color in the case of tyrosine and tyramine is too short for acetone. We have made no attempt to change the conditions so that acetone would give a maximum constant color; but this could, no doubt, be done. We wish merely, to call attention to the fact that with 0.0004 gm. of acetone a color value of 29.6 mm. (F–MO) was obtained when the reaction period was 15 minutes; hence as little as 0.00004 gm. of acetone would give a distinctly perceptible color. This method might, therefore, be useful as a qualitative test for traces of acetone even though further experiments might prove that this method could not be made to give readings constant enough for quantitative work.

It seems hardly necessary to say that these carbonyl-enols can never really interfere with the estimation of tyrosine or tyramine because the carbonyl compounds can always be removed by distillation or evaporation.

Glucose.—Glucose and the other sugars containing a free aldehyde or ketone group would be expected to interfere with the colorimetric estimation of tyrosine because of the yellow color

that these compounds give with sodium hydroxide. When 0.10 cc. of a 5 per cent glucose solution is subjected to the treatment used for estimating tyrosine, a very pale yellow primary color is produced which changes to brown with marked intensification on the addition of sodium hydroxide and then becomes redder when hydroxylamine is added. The color finally obtained is still far too yellow to make a comparison with the (F–MO) standard possible. Tyrosine (0.01 gm.) dissolved in 100 cc. of a 5 per cent glucose solution, produces its own color just as it does in the absence of glucose; but the color finally obtained is the sum of the tyrosine and glucose colors. Thus, of this solution

0.10 cc. had a color value equivalent to 10.3 mm. (F – MO) and
0.20 " " " " " " 19.3 " (F – MO).

The colors obtained in each case contained more yellow than the comparison standard. If these readings are calculated as tyrosine, they are 20 and 13 per cent high, respectively.

Although so high a concentration of glucose would interfere seriously with the colorimetric estimation of tyrosine, lower concentrations are less potent. Thus a tyrosine solution containing 1 per cent of glucose gives a color whose tint and intensity are exactly like those of a solution containing no glucose. *In 1 per cent concentration glucose does not interfere with the estimation of tyrosine.*

Alcohols.—Methyl and ethyl alcohol in 10 per cent concentrations give rise to a color with *p*-phenyldiazonium sulfonate that resembles that produced by tyrosine. The colors are very faint, however, so that a positive interference of about 20 per cent is obtained with 10 per cent concentrations of alcohol. We believe that the pure alcohols do *not* interfere with the colorimetric process and that it is the acetone and acetaldehyde present in the alcohols that produce the color. Ethyl alcohol, that has been freshly distilled over potassium hydroxide augments the color of a tyrosine solution very little even when the alcohol concentration is 10 per cent.

An aqueous tyrosine solution, that has been saturated with amyl alcohol, gives values that are about 5 per cent too high. A chloroform extraction of this liquid *reduces* the reading to about

2 per cent above normal; but does not remove the amyl alcohol completely. Any of these alcohols can, of course, be completely removed by distillation or evaporation; so they can offer no permanent difficulty.

Chloroform, toluene, and ether that has been distilled over sodium, do not interfere with the colorimetric estimation of tyrosine.

Charcoal.—In our earlier work we found that animal or vegetable charcoal adsorbed appreciable quantities of imidazoles and we advised against the use of charcoal in any liquid that was to be tested quantitatively for imidazoles. The adsorption power of charcoal for phenols is far greater than for imidazoles as can be seen from the following data.

5 cc. each of the 1 per cent stock solutions of tyrosine, tyramine, and phenol were separately diluted to 100 cc. and treated with 1 gm. of Pfanstiehl's decolorizing charcoal. After 10 minutes of agitation the liquids were filtered and colorimetric estimations made on the clear filtrates.

Of the tyrosine solution,

0.10 cc. had a color value equivalent to 9.2 mm. (F − MO)

which is equivalent to 0.0115 gm. of tyrosine; hence 0.0385 gm. of tyrosine was adsorbed by 1 gm. of charcoal.

Of the tyramine solution,

0.10 cc. had a color value equivalent to 20.5 mm. (F − MO)

which is equivalent to 0.0256 gm. of tyramine hydrochloride; hence 0.0244 gm. of tyramine hydrochloride was adsorbed by 1 gm. of charcoal.

Of the phenol solution,

1.0 cc. had a color value equivalent to 18.4 mm. (Ph − R)

which is equivalent to 0.0016 gm. of phenol; hence 0.0484 gm. of phenol was adsorbed by 1 gm. of charcoal.

Barium sulfate precipitates do not adsorb phenols from a neutral or acid solution.

SUMMARY.

1. Methods have been devised for the *quantitative colorimetric estimation* of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine.

2. These methods are based upon the well known fact that phenols react with diazonium compounds in alkaline solutions to give colored derivatives. A freshly prepared solution of *p*-phenyldiazonium sulfonate is mixed with a dilute solution of sodium carbonate. A dilute solution of the phenol whose concentration is to be estimated is mixed with the alkaline reagent which gives rise to a primary color that is yellow to red depending upon the character of the phenol.

3. The phenols studied can be divided into three classes.

A. Phenols in which the para position is not occupied by a second substituent.

B. Phenols in which the para position is occupied by a second substituent that does not contain an amino group.

C. Tyrosine and tyramine.

Phenols belonging to Class A (phenol and *o*- and *m*-cresol), couple with great speed and give rise to yellow colors.

Phenols belonging to Class B (*p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids), couple more slowly than those belonging to Class A. The color produced is predominantly red.

Tyrosine and tyramine show an anomalous behavior toward alkaline (Na_2CO_3) *p*-phenyldiazonium sulfonate. An evanescent pink color is produced at first, which fades in 30 seconds to a yellow of inconstant intensity. The simple process employed for the estimation of imidazoles and the other phenols cannot, therefore, be used for the estimation of tyrosine and tyramine. The primary yellow color produced by tyrosine or tyramine is enhanced somewhat by the addition of sodium hydroxide. The colors produced are not directly proportional to the amount of phenol present. If this strongly alkaline liquid is now treated with a small amount of hydroxylamine hydrochloride, a very intense bluish red color is produced whose intensity is directly proportional to the amount of tyrosine or tyramine present (Process II).

4. Tables are given for the direct determination of quantities of these phenols ranging from 0.000001 to 0.00005 gm. The amount of the phenol derivative in any quantity of liquid can then be determined, by multiplication, with an accuracy of from 0.5 to 3 per cent.

5. The alkali salts of the common organic and inorganic acids do not interfere with either of the above two colorimetric processes.

Ammonium salts and amino-acids give an intense yellow color with the process used for the estimation of tyrosine and tyramine (Process II). High values are obtained if these nitrogen compounds are present in sufficient concentration.

Hydrogen peroxide and formaldehyde suppress the color production by tyrosine; hence in the presence of these compounds low values are obtained.

Acetaldehyde, acetone, and acetoacetic acid give rise to a color that is qualitatively identical with that obtained with tyrosine and tyramine. The colors are so intense that the possibility of using this method in the estimation of these carbonyl derivatives suggests itself.

The presence of the ordinary alcohols leads to high readings probably because of the presence of aldehydes or ketones in the alcohols.

1 gm. of vegetable charcoal adsorbs 0.0385 gm. of tyrosine, 0.0244 gm. of tyramine, and 0.0484 gm. of phenol from 100 cc. of an aqueous solution that originally contained 0.05 gm. of the above phenols.

STUDIES ON PROTEINOGENOUS AMINES.

XV. A QUANTITATIVE METHOD FOR THE SEPARATION AND ESTIMATION OF PHENOLS INCLUDING PHENOL, *o*-, *m*-, AND *p*-CRESOL, *p*-OXYPHENYLACETIC, *p*-OXYPHENYLPROPIONIC, AND *p*-OXYPHENYLLACTIC ACIDS, TYROSINE, AND TYRAMINE.

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A study of the products formed from tyrosine by the action of living cells has always been less difficult than the study of the products formed from histidine because the phenols formed can be easily separated into several well defined groups. Experiments on tyrosine have almost invariably been conducted on a large scale, however, because the products formed have had to be identified and estimated by a process of isolation and purification. A purely chemical method, *applicable to small amounts of material, that would effect a quantitative separation into the maximum possible number of groups and that would permit an accurate determination of the constituents of each group without the necessity for an actual isolation of the constituents,* would be superior to any previously described method.

In the preceding communication,[1] two methods were described by means of which small amounts of phenols can be accurately estimated. In that paper, no claims were made for the applicability of the method to mixtures of phenols. It is the object of this report to show that the methods can also be applied to mixtures of phenols and that the exact quantity of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine can be rapidly and accurately determined. The method has been found to be

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1922, l, 235.

applicable, without modification, to liquid media in which bacteria have been allowed to metabolize tyrosine in the presence of salts and glycerol or glucose. In its present form the method is not directly applicable, in its entirety, to more complex liquids such as urine or blood. We hope to modify the method so that it can be applied to such liquids. The underlying principles of the method are as follows:

The simple phenols that are apt to occur associated with living matter can be divided into four groups.

A. Those volatile with steam (phenol and *p*-cresol).

B. Those that will pass into ether from an acidified aqueous solution (*p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids).

C. Tyramine, which can be extracted from an alkaline (sodium carbonate) solution by means of amyl alcohol.

D. Tyrosine, which remains after all of the other phenols have been extracted and which can be determined colorimetrically if imidazoles and amino-acids are not present in high concentrations.

The acidified mixture of phenols is first subjected to a distillation under ordinary pressures. The volatile phenols, phenol or *p*-cresol,[2] pass over quantitatively into the distillate where they can be determined colorimetrically if only *one* of them is present. If these two phenols are present together in nearly equal amounts our method will not estimate them because phenol gives a yellow and *p*-cresol a red color with alkaline *p*-phenyldiazonium sulfonate; and these two colors cannot be estimated separately with a Duboscq colorimeter.

The residue obtained after the volatile phenols have been removed by distillation, is transferred to a glass dish, concentrated to a syrupy consistency on the water bath, and diluted to exactly 25 cc. Of this solution 10 cc. are transferred to an extraction bottle and extracted ten times with ether using 20 cc. for each extraction. *p*-Oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids pass quantitatively into the ether. The combined ether extracts are treated with water (25 cc.) and phosphoric acid (5 drops of the 85 per cent acid) after which the ether is

[2] *o*- and *m*-cresol are also quantitatively volatile with steam. We have not included them in our analytical process because they are not apt to occur in biological fluids under ordinary conditions.

removed by distillation, at first under ordinary pressures and then *in vacuo.* The aqueous solution is then diluted to 100 cc. and the concentration of aromatic hydroxy-acids determined colorimetrically.

Our method does not differentiate between the members of the aromatic hydroxy-acid fraction if they are present together in the same solution. Usually, however, only one member of this group will be present under any given condition. It is possible to tell not only *how much* of a given acid is present but also *which one of the acids is present* by the character of the color produced. The color produced by *p*-oxphenylacetic acid *develops to its maximum intensity within 2 minutes and is slightly brown.*

The color produced by *p*-oxyphenylpropionic acid is also fully developed within 2 minutes; but it contains no brown and it undergoes a sharp change after 2 to 3 minutes of color constancy, that is characteristic for *p*-oxyphenylpropionic acid. The color, which has matched that of the (CR) comparison standard perfectly, suddenly takes on a cloudy appearance and a bluish tint that makes further comparisons impossible.

The color produced by *p*-oxyphenyllactic acid *comes up slowly so that 5 minutes are required to give a color of maximum intensity.* The color finally obtained is slightly more *yellow* than that of the (CR) comparison standard. It is also quite stable: *it does not fade perceptibly for 5 minutes after it has reached its maximum intensity.* This color does *not* undergo a change like that of *p*-oxyphenylpropionic acid.

The acid-containing aqueous liquid, which has been freed from volatile phenols by distillation and from aromatic hydroxy-acids by ether extractions, is carefully treated with solid anhydrous sodium carbonate until the liquid stops effervescing. An excess of sodium carbonate (2 gm.) is then added and the alkaline aqueous liquid extracted six times with amyl alcohol, using 20 cc. for each extraction. The amyl alcohol extracts contain tyramine and the alkaline aqueous liquid contains tyrosine.

A quintuple extraction of the amyl alcohol extracts with N H_2SO_4 removes the tyramine completely from the amyl alcohol. Tyramine can then be estimated colorimetrically in the aqueous acid liquid after neutralization and dilution to 100 cc.

The tyrosine fraction, which contains an excess of sodium carbonate, is transferred to a glass dish, treated with an excess of 37 per cent HCl, and concentrated on the water bath. The crystalline residue obtained is transferred with water to a 25 cc. graduated cylinder and diluted to the mark. Tyrosine is then estimated colorimetrically, in this fraction.

Sections I to IV of this report contain a detailed account of the results of experiments on known solutions of phenol derivatives by which the accuracy of the technique of the method for separating the phenols, described in Section V was experimentally established.

EXPERIMENTAL PART.

I. Tyrosine and Tyramine Not Destroyed by the Prolonged Action upon Them of Hot Hydrochloric Acid and Sodium Hydroxide.

A. Hot 20 Per Cent Hydrochloric Acid.

Tyrosine.—Tyrosine (0.1000 gm.) was mixed with 100 cc. of 20 per cent HCl in a 400 cc. round bottomed Pyrex flask. The solution was boiled for 24 hours over an electrically heated sand bath. The resulting pale yellow liquid was evaporated on the water bath in a glass dish. The residue was treated with water and sufficient hydrochloric acid to give a clear solution. This liquid was then transferred to a volumetric flask and diluted to 1,000 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " 16.0 " (F−MO).

The entire test liquid must, therefore, have contained 0.1000 gm. of tyrosine which is exactly the amount originally introduced.

Continued boiling with 20 per cent hydrochloric acid does not destroy tyrosine to the slightest extent.

An entirely similar experiment was carried out on 0.1000 gm. of tyramine hydrochloride. Of the solution finally obtained

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " 16.0 " (F−MO).

This, by table[3] is equal to 0.1000 gm. of tyramine hydrochloride for the entire test solution which is 100 per cent of the amount originally introduced.

Tyramine is not injured to the slightest extent by continued boiling with 20 per cent hydrochloric acid.

B. Hot 10 Per Cent Sodium Hydroxide.

Tyrosine.—Tyrosine (0.10 gm., 10 cc. of a 1 per cent solution) was mixed with 10 cc. of a 20 per cent solution of sodium hydroxide. The resulting solution was then heated for 10 hours on the boiling water bath in a small, long necked, round bottomed flask. The resulting colorless liquid was transferred, with water, to a 1,000 cc. volumetric flask, neutralized to litmus paper with 5 N H_2SO_4, and diluted to 1,000 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " 16.0 " (F−MO),

which for the entire solution represents 0.10 gm. of tyrosine, 100 per cent of the amount originally introduced.

Tyrosine is not injured to the slightest extent when it is heated for 10 hours with 10 per cent sodium hydroxide.

Tyramine.—An entirely similar experiment was carried out on 0.1000 gm. of tyramine hydrochloride. Of the solution finally obtained

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " 16.0 " (F−MO)

which, by table, is equivalent to 0.10 gm. of tyramine hydrochloride, 100 per cent of the amount originally introduced.

Tyramine is not injured to the slightest extent when it is heated for 10 hours with 10 per cent sodium hydroxide.

II. Phenol and o-, m-, and p-Cresol are Quantitatively Volatile with Steam.

p-Cresol (0.01 gm., 10 cc. of the stock 0.1 per cent solution) was mixed with 140 cc. of water in a 500 cc. distilling flask. The

[3] Tables for converting colorimetric readings into gm. of phenols are given in the preceding article (*J. Biol. Chem.*, 1922, l, 235).

flask was heated with a gas burner through a small hole in an asbestos gauze. The hot vapors were condensed in a spiral glass condenser, the distillate being collected in a 100 cc. graduated cylinder.

Collections.

1. Total volume of the distillate was 50 cc., of which
0.10 cc. had a color value equivalent to 8.0 mm. (CR).
This, by table, is equivalent to 0.0080 gm. of *p*-cresol, 80 per cent of that originally introduced.

2. Total volume of the distillate was 50 cc. of which
0.40 cc. had a color value equivalent to 6.3 mm. (CR).
This, by table, is equivalent to 0.001575 gm. of *p*-cresol, 15.75 per cent of the amount originally introduced.

At this time, 100 cc. of distilled water were introduced into the distilling flask and the distillation was continued.

3. Total volume of the distillate was 60 cc. of which
1.00 cc. had a color value equivalent to 3.0 mm. (CR).
This, by table, is equivalent to 0.00036 gm. of *p*-cresol, 3.6 per cent of the amount originally introduced.

4. The fourth 50 cc. of distillate had no color value.

The total recovery of *p*-cresol in this case was

First 50 cc. contained	0.0080	gm.
Second 50 " "	0.001575	"
Third 60 " "	0.00036	"
	0.009935	"

which is 99.35 per cent of the amount originally introduced.

A second experiment was now carried out to see if a large quantity of *p*-cresol could be recovered quantitatively. This was a duplicate of Experiment 1 excepting that 0.10 gm. of *p*-cresol was used instead of 0.01 gm.

Collections.

1. Total volume of the distillate was 100 cc. The color produced with 0.10 cc. of this liquid was so intense that a direct comparison was impossible; hence 10 cc. of the distillate were diluted to 100 cc. Of this diluted solution
0.20 cc. had a color value equivalent to 9.4 mm. (CR)
which, by table, is equivalent to 0.094 gm. of *p*-cresol for the entire distillate, 94 per cent of the amount originally introduced.

2. Total volume of the distillate was 100 cc. of which
0.20 cc. had a color value equivalent to 6.3 mm. (CR).
This, by table, is equal to 0.0063 gm. of *p*-cresol for the entire distillate, 6.3 per cent of the amount originally introduced.

3. Total volume of the distillate was 50 cc. of which
1.00 cc. had a color value equivalent to 7.2 mm. (CR).
This, by table, is equal to 0.000072 gm. of *p*-cresol, 0.072 per cent of the amount originally introduced..

4. Total volume of the distillate was 50 cc. of which
1.00 cc. had a color value equivalent to 3.5 mm. (CR).
This is equal to 0.000035 gm. of *p*-cresol, 0.035 per cent of the amount originally introduced.

5. The fifth 50 cc. of distillate had no color value.

The total recovery in this case was

First	100 cc. contained	0.094000 gm.	
Second	100 " "	0.0063 "	
Third	50 " "	0.000072 "	
Fourth	50 " "	0.000035 "	
		0.100407 "	of *p*-cresol

which is 100.4 per cent of the amount originally introduced.

Entirely similar experiments were conducted on solutions of phenol, and *o*- and *m*-cresol. In every case between 99 and 100.5 per cent of the phenol was recovered and accounted for, colorimetrically, in the distillate.

Phenol, o-, m-, and p-cresol are completely volatile with steam and they can be estimated quantitatively in the distillates.

III. The Aromatic Hydroxy-Acids Can Be Quantitatively Extracted from an Acidified Aqueous Solution with Ether.

When an acidified aqueous solution containing any of the aromatic hydroxy-acids is extracted ten times with redistilled ether, the aromatic hydroxy-acids pass quantitatively into the ether because *the aqueous liquid no longer gives the slightest color with Pauly's reagent.*[4] If the ether, which must surely contain all of the aromatic hydroxy-acids, is now removed by distillation, and the residue diluted with water to a definite volume, *a colorimetric determination by the usual process either fails to reveal the presence of any phenol or gives values that are far below the theoretical.*

[4] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

We thought, at first, that the aromatic hydroxy-acids might be slightly volatile with steam or ether vapor; but we soon proved that this was *not* true. The other possibility was that the ether contained some imperfectly volatile substances that prevented the phenol from combining with *p*-phenyldiazonium sulfonate in alkaline solution. We surmised that the interfering substance was an oxidation product of the ether, perhaps peroxide in character. To remove this, we agitated a sample of redistilled ether with alkaline permanganate until the permanganate had been decolorized, and redistilled the ether layer. An extraction of the aromatic hydroxy-acids with this *freshly prepared* ether was then carried out. Theoretical values were always obtained with these ether extracts. If, however, the ether was not used for a day or two, low values were again obtained. To avoid the necessity of having to prepare a fresh supply of ether each day, we modified the usual colorimetric procedure as follows.

(1-*X*) cc. of water and *X* cc. of the aromatic hydroxy-acid-containing liquid were treated, for 2 minutes, with 2 cc. of the nitrous acid containing *p*-phenyldiazonium sulfonate reagent. The 1.1 per cent sodium carbonate solution (5 cc.) was then added. This inverse process, which is similar to the process usually used for the qualitative determination of phenols or imidazoles, gave theoretical values both with pure solutions of aromatic hydroxy-acids and with ethereal extracts *even when the ether had not been previously treated with permanganate.* Obviously, then, the free nitrous acid present in the reagent modified the interfering substances so that they no longer prevented the combination between the phenol and the diazonium salt. Fortunately, the nitrous acid did not react with the aromatic hydroxy-acids in such a way as to prevent their coupling with the diazonium salt. *This inverse process should always be used in the estimation of aromatic hydroxy-acids.*

The following experiment will illustrate the method used in the extraction and estimation of the aromatic hydroxy-acids.

p-Oxyphenylpropionic acid (1.00 cc. of the 1 per cent stock solution was mixed in a 35 cc. extraction bottle[5] with 0.2 cc. of 95 per

[5] Any 35 cc. narrow mouthed bottle with a carefully fitted glass stopper will answer the purpose.

cent H_2SO_4 and 9 cc. of water. 20 cc. of specially prepared ether,[6] measured by graduate, were introduced into the bottle, the glass stopper inserted and the liquids vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube and centrifuged for from 1 to 2 minutes. This gave a sharp separation into two layers.

Separation of Ether.—The ether layer was separated from the aqueous layer by means of a device similar to that shown in a previous article.[7] The ether, instead of being drawn into a Squibb funnel, is drawn into a 700 cc. round bottomed flask. Capillary F is, at first, carefully immersed *just below the surface of the ether layer* because the vaporization of the ether as it comes in contact with the large surface of the warm flask usually produces enough pressure to eject some ether back into the extraction bottle which would stir up the aqueous layer if capillary F was too deeply immersed. After the first momentary back pressure, however, capillary F can be gradually lowered until all but a thin film of ether has been drawn into the receiving flask.

The ether not only extracts the aromatic hydroxy-acids but it also reduces the volume of the aqueous layer. It is necessary, therefore, to add sufficient water after each extraction to reestablish the initial volume of 10 cc. It is best to mark the extraction bottle with a carborundum pencil at a level corresponding to a volume of approximately 10 cc.

The above process was repeated nine times so that a total of ten extractions was made.

The aqueous acid liquid gave no Pauly reaction. The ether extracts were then treated with 25 cc. of water and 5 drops of 85 per cent phosphoric acid. The mixture was agitated and subjected to a distillation at first under atmospheric pressure and then *in vacuo* until the ether had been removed entirely. An ebullition tube was *not* used and the distillation was *not* contin-

[6] Commercial ether (800 cc.) is agitated with 50 cc. of alkaline permanganate, such as is used in amino nitrogen determinations by the Van Slyke process, and 50 cc. of water in a 2,000 cc. separatory funnel. After the permanganate has been reduced to MnO_2, the ether layer is poured off and distilled. The redistilled ether so obtained is then ready to use for extractions.

[7] See Fig. 1, Studies on proteinogenous amines. III (Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 526.

ued until all of the water had passed over. The aqueous liquid left in the flask was carefully transferred with water to a 100 cc. graduated precision cylinder and diluted to 100 cc. Of this liquid, 0.10 cc. was mixed with 0.90 cc. of water and 2 cc. of reagent in the right-hand cylinder of the Duboscq colorimeter. After 2 minutes, 5 cc. of the 1.1 per cent sodium carbonate solution were added. The cylinder was transferred to the colorimeter set at 20 mm. and the color compared in the usual manner with that of the (CR) comparison standard. A reading of 3.1 mm. (CR) was obtained. A 0.20 cc. portion of the liquid had a color value of 6.2 mm. This is equal to 0.01 gm. of *p*-oxyphenylpropionic acid, 100 per cent of the amount originally introduced.

Entirely similar experiments were carried out on solutions of *p*-oxyphenylacetic and *p*-oxyphenyllactic acids. 100 per cent recoveries were obtained in every case.

The aromatic hydroxy-acids are quantitatively extracted from an acidified aqueous solution with ether.

IV. Tyramine Quantitatively Separated from Tyrosine by Means of Amyl Alcohol.

A. When a Small Amount of Tyrosine Is Mixed with a Large Amount of Tyramine.

Tyrosine (0.20 cc. of the 1 per cent solution), tyramine (5 cc. of the 1 per cent solution), 0.20 cc. of 95 per cent H_2SO_4, and 5 cc. of water were mixed in a 35 cc. extraction bottle. Anhydrous sodium carbonate was added carefully until the liquid no longer effervesced. Then 2.00 gm. of the carbonate were added and the liquid was agitated until the solid had dissolved. Redistilled amyl alcohol (20 cc., measured by graduate) was introduced into the bottle, the glass stopper inserted, and the liquids were vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube and centrifuged for about 2 minutes. This gave a sharp separation into two layers.

Separation of Amyl Alcohol.—The amyl alcohol layer was separated from the aqueous alkaline layer by means of the device and technique described in a previous article.[7]

The extraction was repeated five times so that a total of 120 cc. of amyl alcohol was used. As in the case of the previously

described ether extractions, the amyl alcohol extracts not only the tyramine but it also markedly reduces the volume of the aqueous layer. It is necessary, therefore, to add sufficient water after each extraction to reestablish the initial volume of 10 cc.

Removal of Tyramine from Amyl Alcohol.—The combined amyl alcohol extracts were extracted five times, in the same Squibb funnel, with 1.0 N H_2SO_4 using 20 cc. for the first and 10 cc. for each of the remaining four extracts. The sulfuric acid extracts were collected in a 100 cc. glass-stoppered precision cylinder and neutralized to litmus paper with 40 per cent sodium hydroxide. The solution was then rendered very faintly acid by adding a few drops of 1.0 N H_2SO_4, transferring to a glass dish, and evaporating on the water bath to remove the amyl alcohol. The crystalline residue was dissolved in water, transferred to a 100 cc. graduated precision cylinder, and diluted to 100 cc. Of the solution so obtained 0.10 cc. had such a high color value that accurate comparisons were impossible; hence 10 cc. of it were diluted with water to 50 cc. Of this diluted solution

0.10 cc. had a color value of 8.0 mm. (F–MO) and
0.20 " " " " " " 16.0 " (F–MO).

This, by table, is equal to 0.05 gm. of tyramine hydrochloride for the entire *original* test solution which is 100 per cent of the amount originally introduced.

The Alkaline Aqueous Liquid (Tyrosine Fraction).—The alkaline aqueous liquid was transferred with water to a glass dish. The dish was covered with a watch-glass. An excess of 37 per cent HCl was added and the liquid concentrated on the water bath. The crystalline residue was dissolved in water with the aid of a few drops of 37 per cent HCl, and diluted to exactly 100 cc. Of this solution

0.50 cc. had a color value equivalent to 8.0 mm. (F–MO) and
1.00 " " " " " " " 16.0 " (F–MO)

which, by table, is equivalent to 0.002 gm. of tyrosine, 100 per cent of the amount originally introduced.

Tyramine is quantitatively extracted from an alkaline (sodium carbonate) aqueous solution by amyl alcohol. Tyrosine, when present in small amounts, does not pass into amyl alcohol from such an alkaline aqueous solution.

B. When a Large Amount of Tyrosine Is Mixed with a Small Amount of Tyramine.

Tyrosine (5.00 cc. of the stock 1 per cent solution), tyramine (0.20 cc. of the stock 1 per cent solution), 0.20 cc. of 95 per cent sulfuric acid, and 5 cc. of water, were mixed in a 35 cc. extraction bottle. Anhydrous sodium carbonate was added carefully until the liquid no longer effervesced. Then 2.00 gm. of the carbonate were added and the mixture agitated until the solid had dissolved. This liquid was then extracted six times with amyl alcohol as described in Section IV, Part A.

Alkaline Aqueous Liquid (Tyrosine Fraction M).—This was treated as described in Section IV, Part A. The solution was finally diluted to 500 cc. of which

0.10 cc. had a color value equivalent to 7.5 mm. (F−MO) and
0.20 " " " " " " " 15.0 " (F−MO)

which, by table, is equivalent to 0.0469 gm. of tyrosine. Since 0.0500 gm. of tyrosine had been originally introduced, *0.0031 gm. of tyrosine must have passed into the amyl alcohol.*

First Amyl Alcohol Extract.—The combined amyl alcohol extracts were extracted with N H_2SO_4 as described in Section IV, Part A. It was necessary in this case, as it will be in most cases, to remove the sulfuric acid with barium hydroxide to avoid the accumulation of a large amount of salts which would interfere with the subsequent treatment. The combined acid extracts were transferred to a 250 cc. Pyrex flask and heated on the water bath. Barium hydroxide (9.0 gm.) was dissolved in 50 cc. of hot water. The resulting solution was added slowly to the acid liquid. The faintly *acid* mixture so obtained was digested on the water bath for 2 hours and filtered through a hard folded filter. The paper and contents were thoroughly washed with hot water. The filtrate and washings were collected in a glass dish, exactly neutralized with sodium hydroxide, and evaporated on the water bath. The solid residue so obtained was transferred with the aid of a few drops of 5 N H_2SO_4 and 10 cc. of water to a 35 cc. extraction bottle. The liquid was treated with 2.00 gm. of anhydrous sodium carbonate. This alkaline solution was then extracted with amyl alcohol in the usual manner which again divides the

material into two fractions; the alkaline aqueous liquid II, which should contain the tyrosine that passed into amyl alcohol at the time of the first extraction, and the purified tyramine fraction which should be free from tyrosine.

Alkaline Aqueous Liquid II (Tyrosine Fraction II).—This was acidified, evaporated, and finally diluted to 100 cc. as in the case of the main tyrosine fraction. Of this solution,

0.50 cc. had a color value equivalent to 11.2 mm. (F−MO) and
1.00 " " " " " " " 22.4 " (F−MO)

which, by table, is equivalent to 0.0028 gm. of tyrosine. Since 0.0469 gm. of tyrosine was recovered in the first, main tyrosine fraction, a total of 0.0497 gm. of tyrosine was accounted for. This is 99.4 per cent of the amount originally introduced.

Purified Tyramine Fraction.—This was extracted with N H_2SO_4 as previously described. The acid was nearly neutralized with 40 per cent sodium hydroxide. The resulting *faintly acid* solution was transferred to a glass dish and evaporated on the water bath. The residue was transferred, with water, to a precision cylinder and diluted to 100 cc. Of this solution,

0.50 cc. had a color value equivalent to 7.9 mm. (F−MO) and
1.00 " " " " " " " 15.8 " (F−MO).

This, by table, is equal to 0.001975 gm. of tyramine hydrochloride, 98.75 per cent of the amount originally introduced.

When the concentration of tyrosine is high, a small amount of it passes into amyl alcohol from an alkaline (sodium carbonate) solution. To free the tyramine fraction from this small admixture of tyrosine it is necessary to conduct a second amyl alcohol extraction on the first tyramine fraction. This slightly longer process with its double amyl alcohol extraction is to be recommended because it makes the determination of tyramine certain and reliable. In most cases it is probably advisable to remove the excess of sulfuric acid from the *final* tyramine fraction with baryta because such solutions would then be practically free from salts and ready for physiological experiments.

V. Separation of Phenols into Four Fractions: Volatile Phenols, Aromatic Hydroxy-Acids, Tyramine, and Tyrosine. The Accurate Determination of One Member of Each Fraction.

The method outlined below is primarily intended to be used in bacterial metabolism studies on tyrosine. We have carried out a sufficient number of such metabolism experiments to be certain that the method gives accurate results. We hope to report these experiments in the near future. Some of the steps in the following experiment were taken, not because they were necessary in this case, but because they were necessary in the metabolism experiments.

A solution containing the following was prepared from the stock solutions; 10 cc. of 1 per cent tyrosine, 4 cc. of 1 per cent tyramine hydrochloride, 4 cc. of 1 per cent *p*-oxyphenyllactic acid, 2 cc. of 1 per cent phenol, 80 cc. of water, and 100 cc. of Nutritive Medium 3.[8]

Filtration.—The clear liquid was forced through a Mandler filter.[9] The flask and filter were washed free from phenols with 200 cc. of water. The filtrate was then treated with 0.50 cc. of 95 per cent H_2SO_4.

Estimation of Volatile Phenols (Phenol).—The filtrate and washings were transferred, with water, to a 1,000 cc. long necked, round bottomed, Pyrex flask. The flask was heated with a gas burner through a small hole in an asbestos gauze. The hot vapors were condensed in a spiral condenser, the distillate being collected in

[8] Nutritive Medium 3 contains:

NH_4Cl	4.00 gm.
KNO_3	2.00 "
KH_2PO_4	8.00 "
NaCl	16.00 "
Na_2SO_4	0.04 "
$NaHCO_3$	8.00 "
$CaCl_2$	0.20 "
Glycerol	80.00 cc.

in a total aqueous volume of 2,000 cc. Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 579.

[9] In bacterial metabolism studies, the hydrogen ion concentration is determined, colorimetrically, on 1 cc. of this filtrate before it is diluted with wash water.

a 250 cc. graduated cylinder. Exactly 200 cc. of distillate were collected of which

0.10 cc. had a color value equivalent to 9.6 mm. (Ph−R) and
0.20 " " " " " " " 19.2 " (Ph−R).

This, by table, is equivalent to 0.01666 gm. of phenol for the entire 200 cc. of test liquid.

The distillation was continued until about 125 cc. of distillate had been collected. This was diluted to exactly 200 cc. Of this solution

0.20 cc. had a color value equivalent to 4.0 mm. (Ph−R) and
0.40 " " " " " " " 8.0 " (Ph−R)

which, by table, is equivalent to 0.0034 gm. of phenol for the entire test solution. In all, 0.02006 gm. of phenol was obtained which is 100.3 per cent of the amount originally introduced. The colors obtained were, in every case, exactly like that produced by pure phenol; hence none of the other phenols volatilized.

Estimation of Aromatic Hydroxy-Acids (p-Oxyphenyllactic Acid).—The liquid left in the flask was carefully transferred, with water, to a glass dish and concentrated on the water bath. The pale yellow syrup was transferred, with water, to a 25 cc. precision cylinder and diluted to exactly 25 cc. We will refer to this as the *test liquid.*

Of this acid test liquid, exactly 10 cc. (measured by pipette) were transferred to a 35 cc. extraction bottle and extracted ten times with specially prepared ether as described in Section III of this paper.

The ether extracts were then treated as described in Section III. The solution finally obtained (volume 100 cc.) was examined colorimetrically for *p*-oxyphenyllactic acid.

0.10 cc. had a color value equivalent to 4.0 mm. (CR)
0.20 " " " " " " " 8.0 " (CR)
0.30 " " " " " " " 12.0 " (CR).

The colors were exactly like that obtained with a pure solution of *p*-oxyphenyllactic acid. This, by table, is equivalent to 0.0400 gm. of *p*-oxyphenyllactic acid *for the entire* 25 cc. *of test liquid,* which is 100 per cent of the amount originally introduced.

Separation of Tyramine from Tyrosine.—The acid liquid left in the extraction bottle, equivalent to 10 cc. of the test liquid, was carefully treated with anhydrous sodium carbonate until the liquid no longer effervesced. Then 2 gm. of the carbonate were added. The mixture was warmed and agitated until the solid had passed into solution. This solution was then extracted six times with amyl alcohol as described in Section IV of this paper.

The Alkaline Aqueous Liquid (Tyrosine Fraction M).—The alkaline liquid left in the extraction bottle was transferred to a glass dish with 100 cc. of water. The liquid was concentrated on the water bath to a volume of about 50 cc., which removed the ammonia completely. The liquid was then treated with 3.5 cc. of 37 per cent HCl, precautions being taken to prevent loss of the solution through spattering. The strongly acid liquid was concentrated on the water bath. The crystalline residue was dissolved in water, with the aid of a few drops of 37 per cent HCl, transferred to a graduated precision cylinder, and diluted to 25 cc. This is the main tyrosine fraction M.

Of this solution 5 cc. (measured by pipette) were diluted to 80 cc. Of this diluted solution

0.10 cc. had a color value equivalent to 7.3 mm. (F–MO) and
0.20 " " " " " " 14.6 " (F–MO)

which, by table, is equivalent to 0.0912 gm. of tyrosine for the entire original test liquid, 91.2 per cent of the amount originally introduced. As one would expect from Experiment B, Section IV, some tyrosine passed into the amyl alcohol from which it will be recovered when the second extraction is carried out (see below).

Amino Nitrogen Determination on Tyrosine Fraction M.—The above tyrosine fraction M (5 cc.) was subjected to an amino nitrogen determination by the Van Slyke method. 1.07 cc. of N_2 were obtained at 25° and 747 mm. which is equal to 0.00729 gm. of nitrogen for the entire test liquid. Tyrosine always gives off about 2 per cent more gas by the Van Slyke process than it should theoretically. We have found this to be invariably true and Van Slyke[10] gives figures that are in perfect agreement with

[10] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 193.

this statement. This nitrogen figure must, therefore, be reduced by 2 per cent before the tyrosine value is calculated. The corrected nitrogen figure is 0.00715 gm.; which is equivalent to 0.00925 gm. of tyrosine, 92.5 per cent of the amount originally introduced. The check between the tyrosine values obtained by these two methods is good.

We wish again to call attention to the fact that this fraction *does not* contain *all* of the tyrosine. A second fraction is obtained later when the first tyramine fraction is reextracted with amyl alcohol. The second tyrosine fraction so obtained usually contains about 0.003 gm. of tyrosine which is too little to estimate by the amino nitrogen method but which is easily determined colorimetrically.

First Amyl Alcohol Extract (Tyramine Fraction I).—This fraction contains all the tyramine together with a small amount of tyrosine. A quantitative separation can be effected by carrying out a second extraction with amyl alcohol in alkaline solution.

The amyl alcohol was, therefore, extracted with N H_2SO_4, the acid removed with baryta, the resulting liquid made alkaline with sodium carbonate and reextracted with amyl alcohol as described in Section IV, Part B.

Second Alkaline Aqueous Liquid (Tyrosine Fraction II).—This was acidified, evaporated, and finally diluted to 100 cc. as described in Section IV, Part B. In this case the ammonia was not removed by evaporation because an amino nitrogen determination is not to be carried out. Of the solution so obtained

0.20 cc. had a color value equivalent to 4.8 mm. (F−MO) and
0.40 " " " " " " 9.6 " (F−MO)

which, by table, is equal to 0.0075 gm. of tyrosine for the *entire original test liquid.* Since 0.0912 gm. of tyrosine was recovered in the main tyrosine fraction M, a total of 0.0987 gm. of tyrosine was accounted for, which is 98.7 per cent of the amount originally introduced.

Purified Tyramine Fraction (Second Amyl Alcohol Extract).—This was extracted six times with N H_2SO_4 as previously described. The acid was nearly neutralized with baryta, the barium sulfate removed by filtration, the filtrate concentrated on the water bath in a glass dish, and the residue dissolved in water and diluted to

exactly 100 cc. as described in Section IV, Part B.[11] Of this solution

0.10 cc. had a color value equivalent to 12.8 mm. (F—MO)

which, by table, is equal to 0.0400 gm. of tyramine hydrochloride, 100 per cent of the amount originally introduced.

SUMMARY.

This paper contains the description of a method by means of which volatile phenols, aromatic hydroxy-acids, tyramine, and tyrosine can be quantitatively separated and estimated. The phenols are determined by a colorimetric process described in the preceding paper. Volatile phenols—phenol, *o*-, *m*-, and *p*-cresol—are distilled off and estimated in the distillate. Aromatic hydroxy-acids—*p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids—are extracted with ether from the acidified aqueous liquid which has been freed from volatile phenols by distillation. The aromatic hydroxy-acids are estimated in the ether extracts. The remaining liquid, which contains all of the tyramine and tyrosine, is made alkaline with sodium carbonate and freed from tyramine by extraction with amyl alcohol. Tyramine is then determined in the amyl alcohol extract; tyrosine is determined in the alkaline aqueous liquid. The separations are quantitative and the colorimetric determinations are accurate to 0.5 to 1.5 per cent.

[11] It is necessary to remove the excess of H_2SO_4 with baryta only if physiological or isolation experiments are to be carried out on this fraction. In other cases it is simpler to neutralize with 40 per cent NaOH as described in Section IV of this report.

SOLUBILITY OF CARBON MONOXIDE IN SERUM AND PLASMA.*

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INTRODUCTION.

In the course of the work of the writers on a method of determination of carbon monoxide in blood, the question arose as to how much of the total gas united with the hemoglobin, and how much merely dissolved in the serum. With the known strong affinity of carbon monoxide for hemoglobin (220 to 300 times as strong as that of oxygen) (1) it would be expected that by far the largest percentage would enter into combination with the hemoglobin, but it seemed of value to investigate just what percentage could be accounted for as being in simple solution. This was especially the case with the Van Slyke method, where the total amount of carbon monoxide in the blood is measured, and not the amount of carbon monoxide hemoglobin. Van Slyke (2) passes over the subject in the case of carbon monoxide; but when dealing with oxygen in the blood (3) has a table of deductions and corrections.

These are estimated on the basis of Bohr's recommendation that the solubility of air in serum is roughly nine-tenths that in water at the corresponding temperature.

On this subject of the solubility of gases in serum and blood, Bohr goes quite into detail (4). He states[1] that the absorption coefficients of oxygen and carbon dioxide in whole blood and that of carbon dioxide in plasma cannot be obtained directly,

* Published by permission of the Director of the United States Bureau of Mines, Washington.

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[1] Bohr (4), p. 62.

because there is also a chemical reaction taking place. But the percentage of deduction (or relation of the coefficient in plasma to that in water) of various gases, when they do not react, is about the same (5). Therefore, when he made determinations and found that the absorption coefficients of oxygen and nitrogen in plasma are 97.5 per cent of those in water, and the absorption coefficient of hydrogen in whole blood is 92 per cent of the figures in water, he felt safe in making the generalization. For later comparison his figures are given; they are calculated by the aid of his deductions.

Coefficient of Absorption in Cc. of Gas (at 0° and 760 Mm.)

	O_2		N_2		CO_2	
	15°	38°	15°	38°	15°	38°
Blood plasma........	0.033	0.023	0.017	0.012	0.994	0.541
Whole blood.........	0.031	0.022	0.016	0.011	0.937	0.511
Blood corpuscles.....	0.025	0.019	0.014	0.010	0.825	0.450

In the case of carbon monoxide, he says[2] simply that the plasma absorbs the gas physically, but proportionately to the tension, and in a slightly lesser amount than would be absorbed by the same volume of water. It seemed worth while, therefore, to make direct determinations instead of relying on estimates. During the progress of this work, conducted at the Pittsburgh station of the Bureau of Mines, some other data were obtained which resulted in certain important conclusions on the use of a table of deductions to correct for the amount of carbon monoxide in the blood, uncombined with the hemoglobin.

Method of Obtaining Serum and Plasma.

As a medium of investigation, beef serum was selected as the one most readily obtainable in quantity; furthermore, most of the related work was being done on beef blood. To supplement this the results were checked on sheep and human sera. Van Slyke and others have assumed that the solubility of carbon monoxide is the same in serum and in plasma. This was also checked

[2] Bohr (5), p. 122.

on beef plasma. The serum in each case was gotten by allowing the blood gathered in the slaughter house to clot quietly in sterile containers, pouring off the serum, and removing remaining corpuscles with a high speed centrifuge running for 10 to 20 minutes. Beef serum has a golden tinge; that of sheep blood is grayer. With human blood, considerable trouble arose from a tendency on the part of the corpuscles to hemolyze; but a few specimens of good serum were obtained. Beef plasma was gotten from blood caught in a sterile container as it poured from the vessels of a freshly killed animal. This was at once poured into 100 cc. bottles containing as a preservative, 0.2 gm. of sodium oxalate, 0.3 gm. of fluoride, and 0.4 gm. of citrate, well mixed. The blood was centrifuged for 3 to 4 hours; the supernatant plasma then pipetted off and further purified by half an hour more in the centrifuge. The oxalated blood separates most readily; best results are gotten by centrifuging within 2 or 3 days after it is drawn from the animal.

Introduction of Carbon Monoxide into the Liquid.

The carbon monoxide used was made by dropping formic acid into concentrated sulfuric acid at 150°C. The evolved gas was washed through potassium hydroxide and stored in a gasometer over water, whence it was delivered as needed, by water displacement. The specimen of serum was allowed to reach thermal equilibrium within a thermostat adjusted to the desired temperature. The carbon monoxide reached this same temperature by being forced through a glass spiral also placed within the thermostat. From here it passed through a bubbler into the serum. 15 minutes were thought sufficient for the saturation of the sample. Frothing was prevented within the specimen tube by the addition of a drop of caprylic alcohol. The effluent gas passed off through a tube into a hood; the serum was thus saturated at atmospheric pressure.

The analysis of the gases dissolved in the serum was performed on the Van Slyke apparatus. All sera saturated below room temperature were kept in ice water, to prevent loss of gas, pending the time when analysis could be made. The method of analysis employed was a modification of that used by Van Slyke in his

determination of oxygen, hemoglobin, and carbon monoxide in blood. It consists in general of drawing off the gases under a vacuum produced by a mercury pump, and of analysis of the evolved substances. The exact technique used in the Bureau laboratory is as follows.

Technique of the Analysis of Gases Dissolved in Blood Serum by the Van Slyke Apparatus.

Before each analysis the apparatus is washed out first with a solution of concentrated ammonia diluted with 3 volumes of water and then twice with distilled water.

5 cc. of serum are measured by pipette into the cup. The end of the pipette is held below the surface of the liquid after starting the delivery, so as to reduce as much as possible exposure of the liquid surface to air. 2 drops of caprylic alcohol are added to the serum, the liquid is drawn into the burette, and the capillary above the stop-cock is sealed with mercury. The apparatus is evacuated and shaken for 2 minutes. The serum is drawn down into the lower bulb and the extracted gases are measured over mercury at atmospheric pressure as described by Van Slyke. The serum is run back into the extraction chamber and shaken further for 1 minute under the same conditions, then the gas volume is read as before. The extraction is repeated until the volume is constant. It is recorded as total gases. The volume contracts a little with standing only a few seconds, as CO_2 dissolved in the small amount of serum on top of the mercury. Oxygen, CO, and N_2 are completely given off by 1 minute shaking in vacuum; the CO_2 comes off much more slowly, requiring usually about 2 minutes.

0.5 cc. of 10 per cent KOH is added to the cup, and is carefully drawn into the pipette, the mercury in the leveling bulb being held slightly below that in the burette. The CO_2 is quickly absorbed. The volume of contraction is noted and the KOH is drawn down into the lower bulb with the serum.

About 5 cc. of potassium pyrogallate[3] solution are next put into the cup and a drop of straw oil quickly added on top of the pyro

[3] 120 gm. of KOH are added to 80 cc. of water, and 50 gm. of pyrogallic acid to 150 cc. of water; 300 cc. of the alkali are then mixed with 40 cc. of the acid solution.

to exclude the oxygen of the air. The "pyro" is drawn into the burette, but not the straw oil, as the latter interferes with gas absorption by solutions, especially CO by Cu_2Cl_2. Oxygen absorption is slow, but is hastened by working the leveling bulb down and up to insure complete contact of gas with the "pyro." When the volume has become constant the "pyro" is drawn down into the liquid in the lower bulb. The volume of gas is read as before. The cup is rinsed out with distilled water, as the pyrogallate left would form a precipitate with the next reagent to be used. A small dropping pipette has been found convenient for removing liquids from the cup.

0.5 cc. of ammoniacal cuprous chloride[4] is now added and carefully drawn through the remaining gases. CO is quickly absorbed and a constant volume obtained almost immediately. The remaining gas is probably nitrogen, though its volume is sometimes higher than would be expected. A complete analysis is made by this method in less than 30 minutes. With practice, readings may easily be made to within 0.005 cc.

In checking up the carbon monoxide determination by this method, the total gases extracted from untreated serum were analyzed as a blank. An analysis made on sheep serum will serve as an example of the results that were obtained.

Volume of total gas.			Volume after absorption by		
1st extraction.	2nd extraction.	3rd extraction.	KOH	Pyro.	Cu_2Cl_2
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
0.270	0.290	0.280	0.065	0.060	0.060

It was found that ammoniacal cuprous chloride does not give contraction in volume of the gases extracted from serum when CO gas is not present.

[4] 400 gm. of cuprous chloride and 500 gm. of NH_4Cl are dissolved in 1,500 cc. of water. For use this is mixed with NH_4OH (sp. gr. 0.90) in proportions of 3:1 (Winkler, L. W., Handbook of technical gas analysis, London, 2nd English edition, 1902, 73).

TABLE I.

Solubility of 100 Per Cent CO in Serum and Plasma in Cc. of Gas per Cc. of Serum.

Temperature.	Beef serum.	Sheep serum.	Human serum.	Beef plasma.
°C.				
15	0.0203	0.0210	0.0209	0.0203
	0.0203	0.0201		0.0195
	0.0203	0.0206		0.0198
20	0.0185	0.0183		0.0181
	0.0176	0.0191	0.0180	0.0181
	0.0181	0.0187		0.0181
25		0.0148		
	0.0157	0.0148	0.0183	0.0173
	0.0166	0.0173	0.0183	0.0164
	0.0161	0.0156	0.0183	0.0169
30		0.0150		
		0.0141		
		0.0158		
	0.0150	0.0169		0.0147*
	0.0140	0.0150	0.0158	0.0147
	0.0145	0.0153		0.0147
37				0.0131
	0.0136			0.0150
	0.0117	0.0153		0.0127
	0.0136	0.0144	0.0143	0.0131
	0.0126	0.0135	0.0142	0.0131
	0.0129	0.0144	0.0142	0.0134

* A sample is shown to illustrate the method of calculating Table I. 0.080 cc. of CO was gotten from a 5 cc. sample, saturated at 30°C., and analyzed at 27°C. and 742 mm.

0.016 = CO per cc. of sample.

$$0.016 \times \frac{273}{300} \times \frac{745 - 22}{760} \times \frac{760}{745 - 32} = 0.0147$$

The first fraction corrects for temperature, the second for the partial pressure of the analyzed gas (the barometric pressure minus water vapor tension at 27°), and the third for the partial pressure under which the gas went into solution (barometric pressure less vapor tension of serum at 30°). There are no figures in the literature for this last, but it is taken as approximately equal to that of water. As a change of 10 mm. in pressure alters the result only 0.0002, which is less than the possible error in reading the apparatus, this approximation seems reasonable.

Determination of Solubility of Carbon Monoxide.

As a satisfactory method has now been developed, a series of determinations was carried out at five different temperatures with the four fluids; *i.e.*, beef, sheep, and human sera, and beef plasma. The figures obtained are shown in Table I.

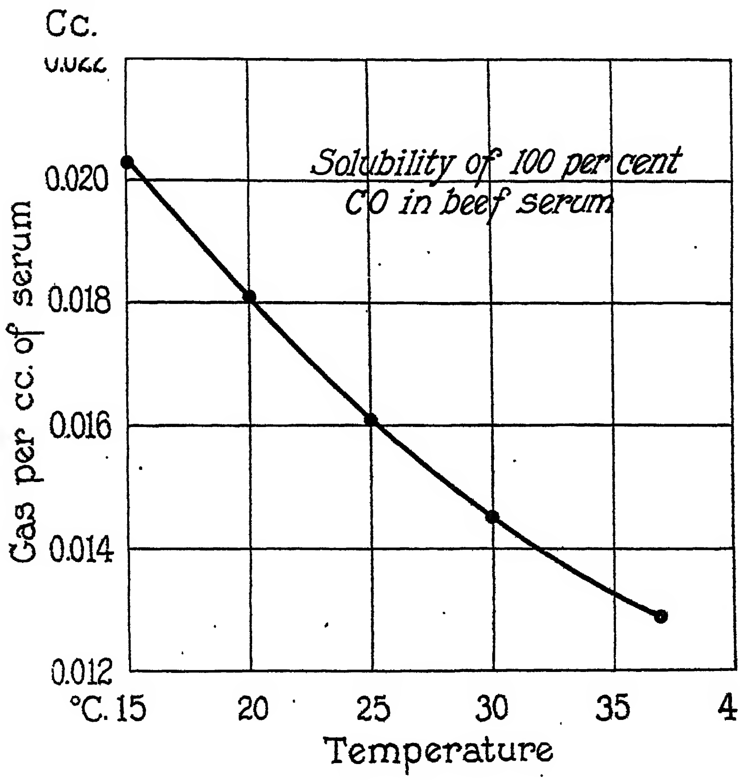

CHART 1.

DISCUSSION.

As would be expected, the figures for the three different sera are practically identical. It is especially interesting to find the results the same with serum and plasma. The accompanying comparison with the table of solubility of carbon monoxide in water (Winkler) gives figures of the same magnitude, but not

quite agreeing with Bohr's hypothesis of a 9:10 relation. In fact, the ratio swings from 8:10 to 7:10 as the temperature rises.

As a check on our method, the same procedure was used in making a determination of the solubility of carbon monoxide in water. Before being used, the water was doubly distilled, then freshly boiled. It was saturated with CO, and then analyzed in the way described above for serum. The results obtained (shown in Table II) are quite in agreement with the figures of Winkler.

TABLE II.

Temperature.	Solubility of 100 per cent CO in beef serum and water.			
	1	2	3	4
	CO per cc. of serum.	CO per 1 cc. water.		Column 1 ÷ Column 3.
		Winkler.*	Author's.	
°C.	*cc.*	*cc.*	*cc.*	
15	0.0203	0.02543	0.0253	0.80
20	0.0181	0.02319	0.0236	0.77
25	0.0161	0.02142	0.0213	0.76
30	0.0145	0.01998	0.0198	0.73
35		0.01877		
37	0.0129		0.0179	0.72
40		0.01775		

* Solubility of CO in water (Winkler, L. W., *Ber. chem. Ges.*, 1901, xxxiv, 1408; *Z. physik. Chem.*, 1892, ix, 171.

The application of these figures of solubility to the calculation of results from the Van Slyke method for the determination of CO and hemoglobin in blood presented some difficulty. As carbon monoxide passes from air into blood, the reaction may be pictured somewhat as follows:

$$\begin{array}{l} \text{CO (in air)} \\ \uparrow\downarrow \\ \text{CO (dissolved)} \rightleftharpoons \text{CO-hemoglobin} \end{array}$$

When the hemoglobin is saturated it might be assumed that the serum is also, and the figures of solubility might be subtracted from the total results. But when the hemoglobin is not entirely saturated with carbon monoxide, is a saturated condition of the

serum to be expected? In view of the strong affinity of the gas for hemoglobin, it would be safe to assume that the tendency in the equation above is to the right. Other and similar questions arise. It may be mentioned that the air in cases of fatal carbon monoxide poisoning rarely contains more than 5 to 10 per cent of the gas (frequently not more than 1 to 2 per cent). From the laws of partial pressure of gases in mixtures, it would be expected that the volume of any one constituent in solution would be proportional to its tension and solubility coefficient in the gaseous mixture with which the solution is saturated.

Saturation of Serum with Carbon Monoxide Mixed with Air.

To ascertain whether this expectation is realized in the case of carbon monoxide in serum, beef serum was saturated at 15 and 37°C., with air which contained 1.13 per cent carbon monoxide (analyses by Bureau gas laboratory), with the results shown in Table III.

The results indicate clearly that the usual laws of partial pressures in gaseous mixtures apply in the case of carbon monoxide in serum, and, therefore, in plasma. The volume of the gas dissolved in 5 cc. of serum, even in as rich a mixture as 10 per cent, is so slight (0.010 cc.) that the experimental error in handling the Van Slyke apparatus with a blood sample would equal or exceed the figure. There is, therefore, in ordinary conditions of CO poisoning, no advantage to be gained in accuracy by subtracting from the total result any figure allowed for carbon monoxide dissolved in serum.

The values obtained are also of importance in their bearing on another question. The figures given by Bohr, cited earlier in this paper in regard to the solubility of gases in plasma and in water, are fundamental in the calculations of hydrogen ion concentrations. In the case of carbon monoxide we have found absorption coefficients whose ratios differ sharply from the 9:10 one Bohr assumed. It seems possible, therefore, that the results of this work may be found to have some influence on the basic figures for hydrogen ion determinations.

TABLE III.

Serum saturated at temperature.	Total gas.	CO_2	O_2	N_2	CO
°C.	cc.	cc.	cc.	cc.	cc.
15	0.135	0.040	0.035	0.060	
	0.120	0.035	0.020	0.055	
37	0.250	0.175	0.025	0.050	
	0.195	0.125	0.020	0.050	

The experiment was repeated with air having 9.8 per cent carbon monoxide (Table IV).

TABLE IV.

Serum saturated at temperature.	Total gas.	CO_2	O_2	N_2	CO
°C.	cc.	cc.	cc.	cc.	cc.
15	0.195	0.090	0.025	0.070	0.010
	0.165	0.060	0.030	0.065	0.010
37	0.120	0.040	0.020	0.050	0.010
	0.110	0.045	0.020	0.045	0.010

The results of the second experiment may be expressed in percentage of the total gas (Table V).

TABLE V.

Specimen.	Per cent of total gas* as:			
	CO_2	O_2	N_2	CO
Air.		18.9	71.3	9.8
Serum at 15°.		23.8	66.7	9.5
		28.6	61.9	9.8
Serum at 37°.		25.0	62.5	12.5
		26.7	60.0	13.8

* Carbon dioxide was disregarded in figuring percentages, as it was absent in the original air bubbled through the serum (although of course from the dissolved CO a small amount of free gas would be found above the solution).

CONCLUSIONS.

The writers have devised a method for the determination of carbon monoxide in serum and plasma. Beef, sheep, and human sera, and beef plasma were saturated with the gas at 15, 20, 25, 30, and 37°C. Compatible figures were obtained. These were of the same magnitude as those of the known solubility of carbon monoxide in water, but only about three-fourths as large as the latter. The method was checked by a determination of the solubility of carbon monoxide in distilled water. The solubility figures in serum and plasma are identical.

Further work was done on the solubility of carbon monoxide by exposing the sera and plasma to mixtures (1 to 10 per cent) of carbon monoxide in air. The amount of this gas dissolved under those conditions was so very small that in calculating results in cases of poisoning under ordinary conditions, no allowance need be made for carbon monoxide dissolved in the serum. The results obtained may have some bearing on hydrogen ion calculations.

In the course of the work the writers have received valuable suggestions from several laboratory and clinical men. They wish especially to express their appreciation for the assistance of Dr. R. R. Sayers, Chief Surgeon of the Bureau of Mines, and A. C. Fieldner, Superintendent of the Pittsburgh Experiment Station who supervised the work; Dr. N. R. Givens of the laboratory staff of the West Penn Hospital, Pittsburgh, for advice in purifying serum and plasma; Mr. Dan Monahan of the Pittsburgh Provision and Packing Company, who very kindly furnished beef and sheep blood for quantity work; Dr. J. C. Burt, who placed the facilities of the state clinic at the disposal of the writers in gathering specimens of human blood; Dr. Stegeman, Professor of Physical Chemistry at the University of Pittsburgh, for advice in connection with several points, and to Dr. D. D. Van Slyke of The Rockefeller Institute, and Dr. Yandell Henderson and Dr. H. W. Haggard of Yale University for suggestions and criticisms.

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THE DETERMINATION OF SODIUM IN SERUM WITHOUT THE USE OF PLATINUM DISHES.

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A few months ago Kramer and Tisdall reported from this laboratory "A simple method for the direct quantitative determination of sodium in small amounts of serum."[1] In this method the sodium is precipitated in platinum dishes. Most clinical laboratories do not possess such dishes. An attempt was, therefore, made to see whether equally good results could not be obtained with dishes of less expensive material.

TABLE I.

Sodium Determinations in Platinum and in Tin Dishes.

Sample.	Sodium per 100 cc. of serum.		Diagnosis.
	Platinum.	Tin.	
	mg.	*mg.*	
Solution "B"	350	347	Known solution; theory, 350 mg.
501	320	325	Normal adult.
523	333	330	" "
"C"	328	326	Epilepsy, adult.
"H"	343	338	Normal adult.
601	320	300	" child.
603	291	295	Scurvy, adult.
"S"	304	309	Rickets.

Four dishes were purchased at a 5 and 10 Cent Store. These are sold in groups of four and are intended for making corn muffins. They are about 9 cm. in diameter and about 3 cm. deep. Parallel determinations on serum were made with these and with platinum

[1] Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 467.

dishes. The results are given in Table I. Samples of a solution of "blood salts" containing known amounts of sodium were also analyzed. The agreement was satisfactory.

CONCLUSIONS.

1. Sodium determinations on serum may be done without platinum dishes.
2. Results obtained when such determinations are done in so called "tin dishes" and in platinum show satisfactory agreement.

THE METABOLISM OF SULFUR.

IV. THE OXIDATION OF CYSTINE IN THE ANIMAL ORGANISM.

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The mechanism of the intermediary metabolism of the more complex amino-acids still remains obscure. It is generally conceded that, as far as concerns the α-amino group, deamination is accompanied by oxidation with the formation of α-keto or α-hydroxy derivatives of the amino-acids. While the manner of oxidation of the cyclic portions of such amino-acids as contain the benzene or other rings is not fully known, it is usually considered that their oxidation is involved in the further oxidation of the keto- or hydroxy-acids, products of deamination. Thus, phenylalanine in moderate amounts is readily and completely oxidized in the body, while phenylpropionic acid (1) is oxidized only to benzoic and cinnamic acids, and eliminated in conjugation with glycocoll without further oxidation. Phenyllactic and phenylpyruvic acids (2), both products of the normal deamination of phenylalanine, are completely oxidized, while β-phenylalanine (3, 4) and phenylserine (3), and their respective deamination products, β-hydroxyphenylpropionic acid and phenylglyceric acid (3) undergo no oxidation of the benzene ring.

Our knowledge of the normal path of oxidation of the sulfur-containing amino-acid, cystine, is even more hazy. The conversion of cystine through cysteinic acid to taurine *in vitro* has been effected by Friedmann (5). However, even if it is to be considered as definitely proved that taurine originates *in vivo* from cystine (6, 7, 8), the fact that the sulfur in taurine itself is oxidized to sulfates with difficulty, if at all (9, 10, 11), in the animal body, makes it improbable that taurine is primarily concerned in the intermediary metabolism of cystine.

The present investigation represents an attempt to determine some of the factors which are involved in the oxidation of the sulfur of the cystine molecule in the animal organism. The methods used in the study of the oxidation of the aromatic amino-acids already discussed, have included a study of the behavior of the products of deamination of the amino-acids in the body. Desaminocystine (12), a derivative of this type, has been prepared, but does not lend itself readily to experiments of the sort undertaken in the present series. We have, therefore, studied the oxidation of the sulfur fraction of the molecule under conditions which prevented deamination of the cystine in order to determine whether, as is probably the case with those amino-acids, which contain the benzene ring, the possibility of oxidation is connected with the cleavage of the amino group and the oxidation of the keto or hydroxy derivatives.

The phenyluramino derivative of cystine has been prepared and its behavior studied in the organism of the rabbit. It has already been shown that uramino-acids are stable in the animal body and do not yield their nitrogen as urea. Salkowski (13) fed the sodium salt of hydantoic acid (uramino derivative of glycocoll) to rabbits and recovered the acid from the urine in a large measure unchanged. Lewis (14) administered the ethyl ester of the same uramino-acid to rabbits and a dog. In no case was there observed an increased elimination of urea although the nitrogen administered was eliminated in the urine. By the preparation of a derivative from the urine evidence was obtained of the presence of the unchanged ester. Rohde (15) was able to recover the uramino derivation of active leucine from the urine of a cat after intravenous injection.

EXPERIMENTAL.

Phenyluraminocystine was prepared from cystine (obtained by hydrolysis of human hair) and phenylisocyanate according to the method of Patten (16). The recrystallized product showed on analysis results which corresponded closely to the theoretical values for sulfur and nitrogen.

The animals used were in all cases rabbits, which were maintained on a diet of milk to which cane-sugar was added to increase the calorific value. Oats were also added in some cases. The

phenyluraminocystine was suspended in water and sufficient sodium carbonate or hydroxide added to effect solution. This solution was either injected subcutaneously or fed through a gastric sound with a portion of the day's allowance of milk. In one experiment (Table V) the phenyluraminocystine was suspended in milk and fed in this form. Cystine was administered either as the sodium salt or as the hydrochloride. No toxic symptoms were noted in any case.

The bladder was emptied by gentle pressure at the same hour daily and the urine thus obtained added to the urine collected from the cage. Total sulfate sulfur was determined according to

TABLE I.

Rabbit A. Male, black. Weight 2.77 kilos. Daily diet: 200 cc. of milk and 10 gm. of cane-sugar.

Date.	N	Total S.	Total SO_4 S.	Unoxidized S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	
Oct. 17	1.136	0.057	0.043	0.014	75.4	24.6	
" 18	0.848	0.036	0.029	0.007	80.6	19.4	
" 19	0.913	0.043	0.035	0.008	81.4	18.6	1.0 gm. phenyluraminocystine *per os* (S = 0.134 gm.).
" 20	1.056	**0.121**	**0.055**	**0.066**	**45.5**	**54.5**	
" 21	0.997	0.057	0.035	0.022	61.4	38.6	
" 22	0.960	0.041	0.035	0.006	84.3	15.7	
" 23	0.943	0.037	0.031	0.006	83.8	16.2	
" 24	1.050	0.048	0.039	0.009	81.3	18.7	1.0 gm. phenyluraminocystine *per os* (S = 0.134 gm.).
" 25	1.055	**0.129**	**0.059**	**0.070**	**45.7**	**54.3**	
" 26	1.006	0.069	0.043	0.023	62.3	37.7	
" 27	1.320	0.073	0.059	0.014	80.8	19.2	
" 28	1.158	0.062	0.053	0.009	86.9	13.1	

Folin; total sulfur by Miss Denis' modification of the Benedict method. Unoxidized sulfur was determined by difference.

The results are presented in Tables I to V. When the sodium salt of phenyluraminocystine was administered *per os* (Tables I and II), there was observed a slight rise in the elimination of total sulfate sulfur, corresponding roughly to about 20 per cent of the sulfur fed in most cases. The greater part of the sulfur of the complex was, however, recovered in the urine as unoxidized sulfur. There was always noted a delayed elimination of part of this "extra" unoxidized sulfur on the day after the administration of the compound. In the first experiment recorded in Table I,

the average daily eliminations of total sulfur and sulfate sulfur during the fore and after periods were 0.040 and 0.033 gm. respectively. During the experimental day and the following day the excretions of total sulfur and sulfate sulfur were 0.178 and 0.090 gm. respectively. This corresponds to the elimination of 0.098 gm. of "extra" total sulfur, of 0.024 gm. of "extra" sulfate sulfur, and of 0.074 gm. of "extra" neutral sulfur. It might be argued that the rise in sulfate sulfur was occasioned by an increased destruction of tissue, resulting from the toxic action of the phenylur-

TABLE II.

Rabbit B. Male, white. Weight 2.2 kilos. Daily diet: 150 cc. of milk and 10 gm. of cane-sugar.

Date.	Total S.	Total SO_4 S.	Unoxidized S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	
Nov. 9	0.026	0.018	0.008	69.2	30.8	
" 10	0.022	0.012	0.010	54.5	45.4	
" 11	0.023	0.013	0.010	58.7	41.3	
" 12	**0.110**	**0.091**	**0.019**	**82.7**	**17.3**	0.501 gm. cystine as sodium salt *per os* (S = 0.134 gm.).
" 13	0.042	0.034	0.008	80.9	19.1	
" 14	0.025	0.018	0.007	72.0	28.0	
" 15	**0.115**	**0.038**	**0.077**	**33.0**	**67.0**	1.0 gm. phenyluraminocystine *per os* (S = 0.134 gm.).
" 16	0.047	0.021	0.026	44.7	55.3	
" 17	0.033	0.018	0.015	54.5	45.5	
" 18	0.038	0.028	0.010	73.9	26.1	
" 19	0.028	0.021	0.007	75.0	25.0	
" 20	**0.132**	**0.041**	**0.091**	**31.1**	**68.9**	0.5 gm. phenyluraminocystine at 10.30 a.m. *per os* and same dose at 2.30 p.m. (S = 0.134 gm.).
" 21	0.072	0.048	0.024	66.7	33.3	
" 22	0.036	0.028	0.008	77.8	22.2	
" 23	0.037	0.030	0.007	81.1	18.9	

aminocystine. The excretion of such an amount of sulfate sulfur from this source should be accompanied by increased nitrogen elimination, which on the basis of a N : S ratio in protein of 14 would amount to over 0.300 gm. However, no appreciable change in the nitrogen excretion was observed. It must be concluded that a partial oxidation of the sulfur has occurred. The total sulfur recovered corresponded to 73.1 per cent of the intake. The detailed figures for the other experiments are very similar to the ones just discussed. In the experiments recorded in Table II,

cystine was fed to afford a control experiment and to demonstrate that the sulfur of cystine administered appeared promptly in the urine as sulfate sulfur. It seemed possible that the slight oxidation of phenyluraminocystine which was observed might be the result of bacterial action in the intestine and that if absorption was facilitated the increase in sulfate sulfur might be lessened. To test out this point the phenyluraminocystine was administered in two doses in order to provide increased opportunity for absorption (Table II). No differences in the degree of oxidation were noted.

TABLE III.

Rabbit C. Male, red. Weight 3.64 kilos. Daily diet: 40 gm. of oats and 150 cc. of milk.

Date.	N	Total S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920	*gm.*	*gm.*	*gm.*	*gm.*	
Dec. 4	1.13	0.067	0.049	0.018	
" 5	1.44	0.082	0.060	0.022	
" 6	1.33	0.069	0.053	0.016	
" 7	1.32	0.078	0.062	0.016	1.0 gm. phenyluraminocystine subcutaneously (S = 0.134 gm.).
" 8	**1.50**	**0.154**	**0.060**	**0.094**	
" 9	1.48	0.062	0.040	0.022	
" 10	1.49	0.078	0.061	0.017	
" 11	1.52	0.098	0.080	0.018	0.501 gm. cystine as sodium salt subcutaneously.
" 12	**1.49**	**0.090**	**0.081**	**0.009**	
" 13	1.31	0.099	0.083	0.016	
" 14	1.52	0.107	0.086	0.021	
" 15	1.49	0.107	0.092	0.015	

When the phenyluraminocystine was introduced parenterally, however (Tables III and IV), no oxidation of the sulfur of the molecule occurred. All of the "extra" sulfur eliminated appeared in the unoxidized sulfur fraction and no increase in sulfate sulfur was evident. The total amount of "extra" sulfur recovered was, however, somewhat less than in the experiments in which the phenyluraminocystine was fed. Cystine when injected in amounts comparable to those of the phenyluraminocystine used did not give rise to any appreciable increase in the unoxidized sulfur fraction. The animal body was able to oxidize completely the sulfur of injected cystine.

In one experiment (Table V) the phenyluraminocystine was fed as a suspension in milk. In this case also no oxidation of the compound took place, and all of the "extra" sulfur was eliminated as unoxidized sulfur. As was to be expected the rate of elimination was slower than in those experiments in which the phenyluraminocystine was fed as the soluble sodium salt. More "extra" sulfur appeared in the urine on the day following the administration than in the preceding experiments.

TABLE IV.

Rabbit D. Female, grey. Weight 1.98 kilos. Daily diet: 150 cc. of milk and 30 gm. of oats.

Date.	Total S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920	*gm.*	*gm.*	*gm.*	
Dec. 13	0.075	0.057	0.018	
" 14	0.072	0.059	0.013	
" 15	0.065	0.052	0.011	
" 16	0.109	0.096	0.015	0.501 gm. cystine as hydrochloride subcutaneously (S = 0.134 gm.).
" 17	0.082	0.067	0.015	
" 18	0.083	0.067	0.016	1.0 gm. phenyluraminocystine subcutaneously as sodium salt (S = 0.134 gm.).
" 19	0.150	0.058	0.092	
" 20	0.075	0.053	0.022	

TABLE V.

Rabbit E. Female, grey. Weight 1.68 kilos. Daily diet: 150 cc. of milk, 10 gm. of sugar, and 30 gm. of oats.

Date.	Total S.	Total SO_4 S.	Unoxidized S.	Remarks.
1921	*gm.*	*gm.*	*gm.*	
Jan. 22	0.037	0.027	0.010	
" 23	0.037	0.026	0.011	
" 24	0.033	0.025	0.008	
" 25	0.043	0.032	0.011	1.0 gm. phenyluraminocystine suspended in milk.
" 26	0.102	0.029	0.073	
" 27	0.067	0.031	0.036	
" 28	0.043	0.029	0.014	
" 29	0.038	0.026	0.012	

The results appear to demonstrate conclusively that, when deamination of the cystine molecule was prevented, the oxidation of the sulfur of the molecule did not take place normally. It

seems probable that in the case of cystine as with the aromatic amino-acids, complete oxidation of the molecule is connected with the deamination process or the further oxidation of the products of deamination. The reason for the slight degree of oxidation when the sodium salt of phenyluraminocystine is administered *per os* is not evident, but we believe this to be the result directly or indirectly of some bacterial action. The study of some other derivatives of cystine in which the amino group is "protected" is in progress.

SUMMARY.

The sulfur of phenyluraminocystine when administered subcutaneously as the sodium salt was not oxidized in the organism of the rabbit, but was eliminated as "extra" unoxidized sulfur. Cystine under the same experimental conditions did not increase the unoxidized sulfur content of the urine. When the sodium salt of phenyluraminocystine was fed to rabbits, a limited oxidation of the sulfur fraction of the molecule, resulting in a slight increase in the elimination of sulfate sulfur occurred, although the greater part of the sulfur administered was recovered in the unoxidized sulfur fraction. Since uramino-acids are not broken down in the organism, these results are believed to indicate that the oxidation of the sulfur of the cystine molecule is connected with the process of deamination or the oxidation of the deamination products.

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THE VITAMINE CONTENT OF MICROORGANISMS IN RELATION TO THE COMPOSITION OF THE CULTURE MEDIUM.

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The Vitamine Content of Yeast.

That yeast is a valuable source of the antineuritic factor was first demonstrated by Schaumann. It has also been claimed that the growth-promoting, water-soluble B substance is abundant in yeast, and it is believed by some workers that these factors are identical, a hypothesis to which we shall return later. Moreover, yeast and yeast extracts have already been introduced into therapeutics, and the latter are also used in food (soup cubes and so forth).

The following observation led us to examine the vitamine content of yeast in regard to the composition of the culture medium.

In my attempts to isolate the antineuritic factor from an aqueous solution of extract of rice polishings, among other methods which I tried, I removed the sugars through the agency of yeast. But the unwished for result was that the medium had lost its antineuritic properties. This experience, in combination with the fact that bakers' as well as brewers' yeast is obtained by cultivating them in media originally containing vitamine, gave rise to the supposition that the yeast cell may not be able to synthesize the vitamine but may take it as such from the medium.

First, we cultivated *Saccharomyces*, isolated from bakers' yeast in vitamine-free media, namely in glucose-peptone broth as well as in a synthetic medium, containing only well known chemical compounds. For this purpose we prepared a solution of

0.5 gm. of NaCl, 0.2 gm. of KH_2PO_4, 0.05 gm. of $CaSO_4$, 0.02 gm. of $MgSO_4$, 5 mg. of $FeSO_4$, 1 mg. of $MnCl_2$, 1 mg. of ZnCl, 0.3 gm. of NH_4Cl, and 5 gm. of dextrose per 100 gm. of water. Henceforth we shall indicate this solution as "synthetic wort." It proved to be suitable for the growth of yeast.

The yeast species which we cultivated at 27°C. in vitamine-free media, proved in experiments on polyneuritic fowls to fail in curative effect. In this connection an old experiment of one of us may be called to mind, according to which polished rice, after its preparation, in the cooked state, into a sweet meat ("tapej") by the addition of Chinese rice yeast ("ragi"), nevertheless remains deficient in the antineuritic factor.

On the other hand, control experiments with the same species of bakers' yeast, cultivated at 27°C. in aqueous solution of extract of rice polishings (sp. gr. 1.045), after washing with physiological salt solution in order to remove the adherent traces of the medium, gave a distinctly positive result. This aqueous extract had been previously divided into two portions, one of which was boiled for a short time only and then filtered and inoculated with the yeast, whereas the other portion was heated before filtering for 1 hour in the autoclave at 120°C. in order to destroy the antineuritic factor. Both of these yielded highly active yeast, but the liquids, separated from the yeast at the end of the fermentation, were found to be inactive.

The same experiments were repeated with other materials, but the result was the same as before. This time we chose a *Saccharomyces* originating from beer yeast, and for media, beer-wort and also a "synthetic wort" so far different from the former that ash of beer-wort, in the same concentration as in beer-wort took the place of the artificial mineral salt mixture, while the reaction of the medium was made slightly acid by the addition of lactic acid.

We took the beer-wort from the brewery in two respective stages of its preparation. The sample of the first stage was heated for a short time only at about 75°C., and proved in experiments on fowls to contain the antineuritic factor. The second sample was boiled for about 2 hours and was hopped. This sample was found to be practically devoid of antineuritic properties. The pure culture from beer yeast which we used in these

experiments, was a so called bottom yeast and the fermentation of the three liquids took place at 6-7°C.

As has already been indicated, the "synthetic wort" yielded yeast without any marked curative power against polyneuritis of fowls whereas the two samples of beer-wort produced a highly, active amount of yeast, the liquids themselves on the contrary proving to be inactive at the end of the fermentation.

It seems, therefore, that yeast not only takes eventually its antineuritic factor as such from the culture medium but that it is not even capable of synthesizing the vitamine unless the medium contains at least the products of decomposition of the vitamine by heating.

In the light of the above mentioned hypothesis this conclusion does not agree with that of Nelson, Fulmer, and Cessna[1] who in experiments on young rats found that yeast can synthesize the growth-promoting, water-soluble B substance in a medium consisting merely of aqueous solution of mineral salts, NH_4Cl, and cane-sugar. These apparently contradictory facts give us reason to doubt whether the antineuritic factor and the water-soluble B substance are really identical. As neither of them has thus far been isolated in an unquestionably pure state, their suggested identity is principally based on the presence or absence of both in the same foodstuffs as also on their conduct towards the same physical and chemical agents. Mitchell,[2] however, drawing attention to the fact that the correlation in all these respects is far from being without exceptions, concludes that in evaluating the data on the occurrence and properties of the two vitamines, there seems to be very good reason for doubling their identity.

Since our experimental results made it probable that the yeast cell may take its antineuritic factor as such from that of the culture medium, although the conclusive proof that the antineuritic factor and water-soluble B are indeed the same has not yet been furnished, the question arose whether from its minute dimensions the yeast cell was able to absorb that factor in a similar way as was already known with regard to charcoal and fullers' earth. In order to decide this question we took advantage of our experience

[1] Nelson, V. E., Fulmer, E. I., and Cessna, R., *J. Biol. Chem.*, 1921, xlvi, 77.

[2] Mitchell, H. H., *J. Biol. Chem.*, 1919, xl, 399.

that yeast, when cultivated in a "synthetic wort," is devoid of the antineuritic factor. Therefore, such yeast was put into vitamine-containing beer-wort, and the mixture shaken for about half an hour at a low temperature. During this time there occurred no noticeable growth of yeast, as could be controlled by sedimenting tests with the hematocrit. After shaking, the yeast was separated from the medium by centrifuging and washing. In experiments on fowls, it did not show any curative effect.

The conclusion may be drawn that the process by which the yeast cell takes its vitamine from the medium is not properly of a physicochemical nature; *i.e.*, an *adsorption*, but a relatively slow, biological one—a *resorption*.

The Vitamine Content of Bacillus coli communis.

According to some authors,[3] various bacterial cultures contain water-soluble B. We did not succeed in confirming these experiments in regard to the antineuritic factor.

A *coli* stock, isolated from the intestines of a fowl, was cultivated at an adequate temperature (42°C.) in a highly active aqueous extract of rice polishings, which had been cautiously sterilized by discontinuous heating at 75°C. After 3 days cultivation the amount of *coli* was collected on a bacterial filter and cleansed by washing. Unlike yeast gained under similar conditions it showed no marked antineuritic properties.

SUMMARY.

Though with some reserve, arising from our insufficient knowledge concerning the composition of the antineuritic factor, we may conclude as follows:

1. The yeast cell can take its antineuritic factor as such from the culture medium. This is not merely an absorption process.

2. The yeast cell is not able to synthesize, in the strict sense of the word, the antineuritic factor, but only to regenerate it after it has been denatured by heating.

3. *Bacillus coli communis*, even after having been cultivated in a medium which contains the antineuritic factor, remains devoid of this vitamine.

4. The antineuritic factor and the growth-promoting, water-soluble B substance are not identical.

[3] Pacini, A. J. P., and Russell, D. W., *J. Biol. Chem.*, 1918, xxxiv, 43. Bierry, H., *Compt. rend. Soc. biol.*, 1919, lxxxii, 307.

A STUDY OF THE EFFECT PRODUCED ON THE COMPOSITION OF MILK BY THE ADMINISTRATION OF CERTAIN INORGANIC AND ORGANIC SUBSTANCES.

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Attempts to modify the composition of milk by experimental means have been numerous and as a rule unsuccessful. To reopen the subject would therefore scarcely appear justifiable except for the fact, that in virtue of the developments in microchemical blood analysis which have taken place during the last decade, we felt ourselves able to approach the problem from a new standpoint. Observations regarding the passage into the milk of drugs administered by mouth have been made for many years, and while such observations are mainly of a qualitative nature, and give as a rule no idea of the amount of absorption, they still indicate the possibility of producing experimentally changes in the mammary secretion by the production of changes in the composition of the blood.

It is now generally conceded that it is possible by dietary measures to influence the concentration of certain of the inorganic and organic non-protein constituents of the blood, whereas in the case of certain other normally occurring constituents forced feeding either produces no demonstrable effect or at most a transitory rise. In the first class urea and phosphates may be mentioned, whereas in the latter striking examples are to be found in sodium chloride and in the salts of calcium.

In view of these facts it would seem that it should in most cases be possible to predict the result of feeding experiments made with the object of producing changes in the composition of the milk (at least as regards a single constituent), provided data are on hand

concerning the possibility of altering the concentration of this specific constituent in the blood.

The experiments described below were undertaken with the purpose of obtaining experimental proof of the validity of the above hypothesis. In this paper are given the results obtained by the administration of urea and calcium chloride.

Our experimental methods were as follows:

An appropriate amount of the substance whose absorption was to be studied was dissolved in about 300 cc. of water and poured down the animal's throat without the use of stomach tube. All samples of blood were taken by venepuncture from the external jugular vein.

The analytical methods used were as follows:

For Milk.

Urea.—By the urease method according to the technique described by Denis and Minot (1).

Calcium.—By Lyman's (2) method, slightly modified, as an extended experience has shown us that in experimental work better results are obtained if several standards of varying strength are provided instead of the single standard recommended by Lyman.

For Blood.

Urea.—By the method of Folin and Wu (3).

Calcium.—By the method of Lyman (2).

Absorption of Urea.

The ability of the body rapidly to absorb ingested urea is now too well known to require comment. It has been shown by Marshall and Davis (4) that when urea is injected intravenously it is stored in the muscles and in the various organs of the body in amounts approximately equal to the concentration found in the blood. Of recent years much experimental work has been carried out on the effects of high and low protein diets on the concentration of blood urea; in general the results of this work lead us to believe that it is possible, even in subjects with normal kidney function to increase the concentration of blood urea by relatively short

periods of high protein feeding, and to decrease it by the use of low nitrogen diets. It was also shown some years ago by Denis and Minot (5) that by the administration of diets high in protein it was possible to obtain from cows, milk of high urea content, whereas the same animals when fed on low protein diets produced milk containing relatively small amounts of urea. These studies were unfortunately not accompanied by observations of blood urea. In Experiments 1, 2, and 3, the results of which are tabulated in Tables I, II, and III we have studied the relative effects of large doses of urea on the blood and milk.

TABLE I.

Absorption of Urea.

Experiment 1.—Goat 1, weight 31.8 kilos. An old animal, whose average yield of milk for 24 hours was about 150 cc.

Time.	Urea administered.	Volume of milk.	Urea N per 100 cc. of milk.	Increase of urea N over normal.
1920	*gm.*	*cc.*	*mg.*	*per cent*
May 24, 8.15 a.m.		65	17.8	
9.00 "	5			
10.00 "	5			
11.00 "	5	15	19	6.9
12.00 m.	5			
1.00 p.m.		7	26	46.0
3.00 "		3	95	433.7
May 25, 9.00 a.m.		37	29	62.9

As will be seen such treatment causes a rapid rise in the urea nitrogen fraction of both blood and milk. If we disregard the results of Experiment 1 in which no blood examinations were made, it will be noted that the urea content of the milk had increased to more than 30 per cent of its former value in 1 hour in Experiment 2, whereas in Experiment 3 a similar result was obtained in these specimens of milk taken 3 hours after the administration of urea. A similar relation exists between the two experiments as regards the final concentration of urea attained. In Experiment 2 the maximum figures were obtained 6 hours and 45 minutes after the administration of the initial dose, the rise being represented by an increase of 163 per cent in the milk and 206 per cent in the

plasma, whereas in Experiment 3 after 11 hours the rise in the urea nitrogen of the milk was represented by an increase of 97 per cent.

TABLE II.

Absorption of Urea.

Experiment 2.—Goat 2, weight 22.5 kilos. A young animal in the first month of lactation. The average yield of milk for 24 hours was about 1 liter.

Time.	Urea administered.	Volume of milk.	Urea N per 100 cc.		Increase of urea N over normal.	
			Milk.	Plasma.	Milk.	Plasma.
1920	*gm.*	*cc.*	*mg.*	*mg.*	*per cent*	*per cent*
June 9, 9.00 a.m....		600	14.4	15.0		
9.15 " ...	5					
10.15 " ...	5					
11.15 " ...	5	96	19		31.9	
12.15 p.m....	5					
1.30 " ...		40	37		15.69	126.0
3.15 " ...		40	38		163.8	206.6
10.00 " ...		170	25.5		77.0	
June 10, 8.30 a.m....		420	12.4			

TABLE III.

Absorption of Urea.

Experiment 3.—Goat 3, weight 36 kilos. An old animal in the first month of lactation. The average 24 hour yield of milk was about 700 cc.

Time.	Urea administered.	Volume of milk.	Urea N per 100 cc.		Increase of urea N over normal.	
			Milk.	Plasma.	Milk.	Plasma.
1920	*gm.*	*cc.*	*mg.*	*mg.*	*per cent*	*per cent*
June 22, 6.00 a.m....		350	13.4	20		
6.10 " ...	5					
7.00 " ...	5					
8.10 " ...	5					
9.10 " ...	5	65	17.6		31.3	
12.00 m......		30	19	30	44.7	50.0
3.00 p.m....		44	19	31	41.7	55.0
5.00 " ...		50	26.4		97.0	
June 23, 9.00 a.m....		320	29		116.4	
5.00 p.m....		200	15.3		14.1	
June 24, 9.00 a.m....		310	17		27.5	

Absorption of Calcium Chloride.

Feeding experiments made with the object of modifying the calcium content of milk have usually been unsuccessful (6). Similar negative results have been the reward of investigators who have attempted to increase the calcium content of the blood by the administration of calcium salts (7). It is not surprising, therefore, that our attempts to change the calcium concentration of the milk by feeding large doses of calcium chloride (as described in the tabulation of the results obtained in Experiments 7, 8, and 9, Tables IV, V, and VI) should also have yielded negative results as regards both milk and blood.

TABLE IV.

Absorption of Calcium Chloride.

Experiment 7.—Goat 1.

Time.	$CaCl_2$ administered.	Volume of milk.	Per 100 cc. of milk.		Increase.	
			Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*per cent*	*per cent*
May 29, 9.00 a.m....		85	15.9	156		
9.30 " ...	2					
10.30 " ...	2		158	156	0	0
11.30 " ...	2					
12.30 p.m....	2	37	158	180	0	15.3
3.30 " ...		13	158	180	0	15.3

In Experiments 7 and 8 unmistakable increase in the chloride concentration in the plasma and milk was observed. In Experiment 9, however, this increase was confined to the plasma, no change being noted in the milk.

As it occurred to us that our negative results in the case of these experiments in which calcium chloride was administered by mouth might be due to slow absorption of this salt from the stomach and small intestine we performed a final experiment in which 1.87 gm. of calcium chloride contained in a volume of 75 cc. were injected intravenously. The duration of the injection was 3 minutes. The plasma from a sample of blood taken 17 minutes after the end of the injection showed an increase in the calcium content of 150 per cent, 3 hours later the plasma calcium was still 48 per cent above the initial figure. Even with this unmistakable

increase in the calcium concentration of the blood, however, no increase was noted in the calcium concentration of several samples of milk removed at intervals during the day.

TABLE V.

Absorption of Calcium Chloride.

Experiment 8.—Goat 2.

Time.	$CaCl_2$ administered.	Volume of milk.	Per 100 cc.				Increase.			
			Milk.		Blood.		Milk.		Blood.	
			Ca	Cl	Ca	Cl	Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
June 29, 8.50 a.m......		320	160	140	8.6	354				
9.00 "	4									
10.00 "	4									
11.00 "	4									
12.00 m.........	4	44	161	164	8.5	360	0	17.1	0	1.6
3.00 p.m......		43	160	169	8.6	380	0	20.7	0	10.1
June 30, 9.00 a.m......			160			361	0			1.6

TABLE VI.

Absorption of Calcium Chloride.

Experiment 9.—Goat 3.

Time.	$CaCl_2$ administered.	Volume of milk.	Per 100 cc.				Increase.			
			Milk.		Plasma.		Milk.		Plasma.	
			Ca	Cl	Ca	Cl	Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
June 29, 8.40 a.m......		420	163	169	8.7	360				
9.00 "	4									
10.00 "	4									
11.00 "	4									
12.00 m.........	4	72		169	8.6	402		0	0	10.1
3.10 p.m......		60	164	180	8.7	432	0	0	0	20.0
June 30, 9.00 a.m......		380	164	170	8.6	360	0	0	0	0

The results of the experiments described above would appear to lend support to the hypothesis outlined in the opening paragraphs of this paper. If we believe that the mammary tissue may act

as a temporary storage place for certain substances that are not rapidly excreted our work would suggest that both the chlorine ion and urea may be found in increased amounts in the milk when their concentration in the plasma, and probably also in the mammary tissue, rises to a high level. The calcium ion on the other hand apparently acts in an entirely different manner. The cause of this difference in behavior may be ascribed to the relative toxicity of calcium with the resultant lowering of the dosage, or to the fact that calcium cannot be retained in the mammary tissue but is

TABLE VII.

Absorption of Calcium Chloride.

Experiment 10.—Goat 3.

Time.	$CaCl_2$ injected.*	Volume of milk.	Per 100 cc.				Increase.			
			Milk.		Plasma.		Milk.		Plasma.	
			Ca	Cl	Ca	Cl	Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
July 7, 9.30 a.m.		360	200	164	128	350				
10.35 "	Injected 1.87 gm. $CaCl_2$.									
10.55 "					32	400			150	14
11.55 "		55	200	175			0	6.6		
1.50 p.m.		35	196	180	19	350	0	9.7	48	0
4.10 "		30	200	173	13	350	0	6.4	1.4	0
July 8, 9.10 a.m.		200	201	180			0	9.7		

* At 10.30 injected into the external jugular vein 75 cc. of a 25 per cent solution of calcium chloride.

excreted almost immediately by the intestine and kidney. In view of the unmistakable increase in the chlorine concentration of the milk of the animals receiving calcium chloride we feel inclined to accept this latter view.

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THE THERMOSTABLE ACTIVE AGENT OF PIG'S PANCREAS.

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More than a year ago the writer stated that yeast nucleic acid is decomposed into its nucleotides by a boiled aqueous extract of pig's pancreas without giving rise to any increase in the acidity of the solution; and the crucial bearing of this circumstance upon the chemical constitution of yeast nucleic acid was discussed in detail.[1]

The context of the article and of other writings on the subject indicated clearly that the word "acidity" was intended to mean "titratable acidity" (not necessarily hydrogen ion concentration)[2] and the implication was definite that titrations had been made. In the experimental part (which was not otherwise complete) no specific description was given of a titration, the principal description being of an experiment dealing only with hydrogen ion concentration. This experiment Levene[3] now criticizes in the following words:

"The second experimental proof of Jones' theory is the following: By a pancreas enzyme, yeast nucleic acid was cleaved to its nucleotides. At the starting point of the experiment the hydrogen ion concentration of the reacting mixture was brought to pH = 6.4, and at the end of the experiment there was no apparent change of the color of the indicator added to

[1] Jones, W., *Am. J. Physiol.*, 1920, lii, 203.

[2] See Jones, W., Monograph on biochemistry, London, 1920, 47, "the increased acidity caused by the decomposition of two grams of nucleic acid should require about 8 cc. of tenth normal sodium hydroxide for neutralization toward phenolphthalein. There is no doubt about this. The nucleotides imitate the conduct of phosphoric acid toward alkalis and indicators."

[3] Levene, P. A., *J. Biol. Chem.*, 1921, xlviii, 119.

the original solution. Hence the author concludes that no acid radicles could be liberated as the result of the hydrolysis. The reasoning is not correct. According to either theory, nucleic acid is a polyphosphoric acid and when brought to a pH = 6.4, it possesses considerable buffer effect. Furthermore, each nucleotide is a comparatively weak acid and when liberated does not affect the hydrogen ion concentration of the buffer very markedly. Since the dissociation constant of the nucleotides has not been measured, it is not possible to express the reaction in quantitative terms. Experimentally, however, we convinced ourselves that when a solution of guanosinphosphoric acid is brought to a pH = 6.4, it stands the addition of an equal volume of a solution of free guanosinphosphoric acid of the same concentration before any change of color of the indicator can be noticed. Taking further into consideration the fact that a solution of nucleic acid is not perfectly colorless, that an extract of the pancreas always contains a considerable quantity of phosphates and also is not colorless, one easily realizes that the argument of Jones carries but little weight."

This criticism would be more plausible if the following were ignored:

1. The pale yellow color of a boiled pancreas extract matches well the alkaline color of the indicator used (brom-cresol). The change of this indicator from its purple color to yellow can be detected just as well in a pale yellow solution as in distilled water when a proper arrangement for the observation is made.

2. A 2 per cent solution of Merck's yeast nucleic acid is scarcely colored. But aside from this, the specimen of yeast nucleic acid that we have been using in this laboratory is snow-white and forms a colorless 2 per cent solution. A method will be described later for its preparation from yeast and from the various commercial sources of yeast nucleic acid.

3. All four of the nucleotides are strong enough acids to turn methyl orange.

4. Levene's experiment with guanosinphosphoric acid has been repeated with both adenine nucleotide and guanine nucleotide. The results obtained are strikingly different from the result given by Levene.[4] 15 cc. of a 7 per cent solution of adenine nucleotide in hot water were cooled, colored with brom-cresol, and brought to a hydrogen ion concentration of pH = 6.4. The purple solution was divided into two equal parts, one of which

[4] Levene does not state what indicator he used. This is of vital importance.

was used for comparison while the other was carefully treated with a 7 per cent solution of adenine nucleotide. The first drop produced a color change that could doubtfully be detected; the second drop produced an unmistakable change in the color of the indicator; the third drop so changed the indicator that its predominant color was yellow. Essentially the same results were obtained with guanine nucleotide.

5. No *titratable acidity* is produced when yeast nucleic acid is decomposed into its nucleotides by the action of boiled extract of pancreas, as the following experiments will show.

A boiled aqueous extract of pig's pancreas was prepared according to the directions previously given[1] and 3 gm. of yeast nucleic acid were dissolved in 150 cc. of the warmed extract.[5] This solution, in which the presence of the nucleic acid could be shown even after excessive dilution, was quickly cooled to the room temperature, placed in a burette, and compared with a tenth normal solution of sodium hydroxide using phenolphthalein as an indicator with the usual precautions. The results of ten titrations given in Table I show that for 5 cc. of the nucleic acid solution close to 5.60 cc. of the alkali were required for neutralization toward phenolphthalein.

The remainder of this nucleic acid solution was preserved with a few drops of chloroform and allowed to digest in the thermostat at 38° for 24 hours. At the end of this time the nucleic acid had entirely disappeared, having been converted into its nucleotides. After cooling to the room temperature the solution was placed in a burette and again compared with the tenth normal solution of sodium hydroxide, using phenolphthalein as an indicator with the usual precautions. The results of eight titrations given in Table I show that for 5 cc. of the digested solution close to 5.75 cc. of the alkali were required for neutralization toward phenolphthalein.

Weighed portions of adenine nucleotide were then added to 5 cc. portions of the digested fluid and the product was titrated as before. The titratable acidity was found increased by the theoretical demand.[6]

[5] The nucleic acid dissolves readily in the warmed extract without the addition of alkali.

[6] The experiment here reported was done after Levene's article appeared, but the results do not materially differ from those of numerous older and recent experiments.

The two formulas which have been proposed in turn by Levene for yeast nucleic acid and the one proposed by Thannhauser and Sachs[7] demand the production of titratable acidity in each of the ten experiments corresponding to 4.60, 2.31, and 5.25 cc. of tenth normal sodium hydroxide, respectively. But no titratable acidity at all was found, although adenine nucleotide exhibits its theoretical titratable acidity in a boiled aqueous extract of pig's pancreas.

It is true that no experimental evidence exists to show the points at which the individual nucleotide groups of yeast nucleic

TABLE I.

Before digestion (nucleic acid).			After digestion (nucleotides formed).			
Extract + nucleic acid used.	Alkali required.	Alkali calculated for 5 cc. of extract.	Extract + nucleotides formed.	Adenine nucleotide added.	Alkali required.	Alkali calculated for 5 cc. of extract.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*cc.*	*cc.*
3.42	3.80	5.55	3.17		3.55	5.60
3.81	4.27	5.60	3.81		4.19	5.50
3.60	4.07	5.65	3.30		3.63	5.50
3.12	3.43	5.50	4.00		4.56	5.70
4.18	4.68	5.60	4.12		4.70	5.70
3.97	4.49	5.65	3.30		3.70	5.60
3.51	3.90	5.55	3.21		3.53	5.50
3.70	4.07	5.50	3.60		3.96	5.50
4.07	4.60	5.65	3.19	50	6.25	
4.13	4.71	5.70	3.34	50	6.42	
4.07	Used for comparison.		3.18	Used for comparison.		
Mean		5.60	Mean			5.75

acid are joined to one another; but there exists abundant experimental evidence to show the points where they are *not* joined. The nucleotide linkages do not involve any one of the phosphoric acid groups and no formula for yeast nucleic acid can be accepted in which this kind of nucleotide linkage is assumed. The carbohydrate formula that I have used is arrived at only by exclusion and is intended specially to indicate the points where the nucleotide linkages *do not exist.*

[7] Thannhauser, S. J., and Sachs, P., *Z. Physiol. Chem.*, 1921, cxii, 189.

In the same article Levene[3] criticizes another kind of experimental evidence that I have adduced, and in the following words:

"The curve expressing the rate of hydrolysis of yeast nucleic acid is identical with that of a mixture of the four nucleotides. Accepting the experiment as correct, what does it demonstrate? It proves that the union between individual nucleotides is more labile than that between the phosphoric acid and the carbohydrate in each nucleotide. It is then self-evident that the first step in the hydrolysis of the nucleic acid molecule is the formation of four nucleotides. The further progress of hydrolysis of the nucleic acid is the same as of four nucleotides."

I raised this question myself long ago and dealt with it in the following words.[8]

"It is of course possible to draw other conclusions that can be adjusted to the facts, but they all involve the assumption of curious coincidences and compensations which would cause phosphoric acid to be liberated with equal ease from different kinds of linkage, or that the liberation of phosphoric acid from one kind of linkage is excessively slower than from another. After careful consideration of such matters, we believe we have drawn the correct conclusion."

The contents of the present article show that this statement did not exhibit poor critical judgment.

In Levene's article[3] is contained the following unfortunate sentence.

"It is peculiar that Jones, in the latest edition of his monograph, in discussing the theories of the constitution of yeast nucleic acid, does not at all refer to the theory of the present writer."

It is not peculiar. I knew at the time what is contained in the present article. Had I known what is contained in Levene's recent communication,[3] I should not have referred to his theory of the constitution of *thymus* nucleic acid.

CONCLUSION.

The existence in the pancreas extract of an easily detected thermostable agent which decomposes yeast nucleic acid only as far as its nucleotides, is more interesting in its physiological significance than for any light that it throws upon the chemical

[8] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 126.

constitution of nucleic acid. The first alteration that yeast nucleic acid undergoes in its decomposition by tissue extracts is assumed to be the production of nucleotides. How then does it happen that extract of pig's spleen and of other tissues, that do not contain the thermostable agent in question, can nevertheless bring about the progressive decomposition of nucleic acid with the formation of free phosphoric acid and free purine bases?

It would appear either that there are two ferments which can decompose nucleic acid into its nucleotides, one of which is destroyed by heat, or that the decomposition of nucleic acid by tissue extracts does not proceed along conventional lines. Examination of the matter is now proceeding.

A RAPID COLORIMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF THE INORGANIC PHOSPHORUS IN SMALL AMOUNTS OF SERUM.

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Principle of the Method.

The principle of the method consists in the precipitation of the phosphorus, in a trichloroacetic acid extract of serum, as strychnine phosphomolybdate, the isolation of the precipitate by the use of a centrifuge and small quantities of water, and the subsequent development of a brilliant green color produced by the reduction of the molybdenum present in the precipitate. The reduction is accomplished by the use of potassium ferrocyanide and HCl.

The Method.

Precipitation of Protein.—1 cc. of serum is transferred to a 15 cc. centrifuge tube and to this are added 5 cc. of a 6 per cent solution of trichloroacetic acid. The mixture is thoroughly mixed with the aid of a glass rod and allowed to stand for 4 minutes. It is then centrifuged for 4 to 5 minutes at about 1,500 revolutions per minute and the supernatant fluid poured off.

Precipitation of Phosphorus with the Strychnine Molybdate Reagent.—5 cc. of the supernatant fluid are measured into an ordinary 15 cc. graduated centrifuge tube, the outside diameter of which is 6 to 7 mm. at the 0.1 cc. mark. Water is added to bring the volume to 6 cc., followed by 2 cc. of the strychnine molybdate reagent which should be added drop by drop, and the tube shaken three or four times during the procedure. The contents of the tube are then thoroughly mixed by holding the tube at the upper

end and tapping the lower end with the finger giving it a circular motion. The contents are allowed to stand for 10 minutes during which time they are thoroughly mixed twice as outlined above.

Washing of Precipitate.—After the 10 minutes has elapsed the tube is centrifuged at 1,500 revolutions per minute for 3 minutes, the supernatant fluid is poured off and the mouth of the tube wiped with a dry cloth. 3 cc. of water are allowed to run down the sides of the tube which removes any adherent supernatant fluid. The residual supernatant fluid (about 0.1 cc.) is thoroughly mixed with the added water by tapping the lower end of the tube with the finger giving it a circular motion, while the precipitate is disturbed as little as possible. The mixture is centrifuged for 1 minute at 1,500 revolutions per minute, the supernatant fluid is poured off and the above procedure repeated, making two washings in all.

Development of Color.—After the final supernatant fluid has been removed, 2 cc. of a 1 per cent solution of NaOH are added and the contents mixed with the aid of a glass rod. This causes all the precipitate to go into solution. Water is added to 10 cc. and the contents are transferred to a 100 cc. glass-stoppered volumetric flask. Traces of the solution remaining in the centrifuge tube are washed into the flask by means of two lots of 10 cc. of water, so that the total volume of fluid in the flask is 30 cc. 20 cc. of a 20 per cent solution of potassium ferrocyanide are then added, followed by 10 cc. of concentrated HCl. The flask is inverted two or three times and allowed to stand 10 minutes. Water is added to 100 cc., the contents are thoroughly mixed, and the color is read in the colorimeter against the standard.

Preparation of the Standard.—1 cc. of a solution of KH_2PO_4 containing 5 mg. of P per 100 cc. (219.3 mg. of KH_2PO_4 (Merck) in 1,000 cc.) is measured into a graduated centrifuge tube, which contains 5 cc. of water, and the contents are thoroughly mixed. 2 cc. of the strychnine molybdate reagent are then added drop by drop. This step, and the washing of the precipitate and the development of the color, are carried out at the same time and in the same manner with both the standard and the unknown.

The Volume of the Precipitate.—The amount of precipitate obtained in the standard solution after it is centrifuged is almost exactly 0.1 cc. of volume. If the amount of precipitate obtained

in the unknown is 0.2 cc. or more, its solution (in 1 per cent NaOH) should be made up to a definite volume in the centrifuge tube and an aliquot taken which would contain approximately 0.1 cc. of the precipitate. If the amount of precipitate obtained in the unknown is about one-half the amount in the standard, its solution should be made up to 5 cc. and transferred to a 50 cc. volumetric flask with the use of two lots of 5 cc. of water. In all the subsequent steps the volumes used should be halved.

Calculation.—When the unknown is made up to 100 cc. and the standard solution is set at 20 in the colorimeter the calculation is as follows:

$$\frac{20}{\text{Unknown}} \times 6 = \text{mg. of P per 100 cc. of serum.}$$

When the unknown is made up to 50 cc. the result is divided by 2.

Preparation of the Strychnine Molybdate Reagent.

Solution A is prepared by dissolving 50 gm. of ammonium molybdate in 150 cc. of warm water. If not clear this solution should be filtered.

Solution B consists of 2 volumes of concentrated HNO_3 and 1 volume of water.

Solution C is prepared by pouring 1 volume of Solution A into 3 volumes of Solution B.

Solution D consists of strychnine nitrate 7.5 gm., water to 500 cc. The water may be warmed to facilitate solution.

1 volume of Solution D is poured into 3 volumes of Solution C. This constitutes the strychnine molybdate reagent. The reagent should stand 24 hours before it is used. It will keep for at least 1 month. After the reagent has stood for 1 or 2 days a slight precipitate forms and when this occurs it should be filtered. 2 cc. of the reagent will precipitate 0.2 mg. of P.

Protocols.

The development of the color on the addition of potassium ferrocyanide and HCl is in direct proportion to the amount of molybdenum present, as long as that amount does not exceed double the quantity, or is not less than half the quantity present

in the standard. A solution of strychnine phosphomolybdate was prepared so that 2 cc. contained approximately the amount of precipitate obtained from 0.05 mg. of P (1 cc. of the standard solution). 1, 2, and 4 cc. of this solution were placed in 100 cc. volumetric flasks, the volumes made up to 30 cc. with water, and potassium ferrocyanide and HCl added. After 10 minutes the flasks were made up to volume and the color was read in the colorimeter with the flask containing 2 cc. of the solution taken as the standard at 20. Reading obtained with 1 cc. of solution

TABLE I.

Estimation of Known Amounts of Phosphorus in a Solution of KH_2PO_4.

Amount of solution.	Quantity of P.	Volume of precipitate after centrifuging.	Size of flask.	Amount of potassium ferrocyanide added.	Amount of HCl added.	Reading.	Theory.	Error.
cc.	*mg.*	*cc.*	*cc.*	*cc.*	*cc.*			*per cent*
0.25	0.0125	Less than 0.05.	50	10	5	40.8	40.0	−2
0.5	0.025	About 0.05.	50	10	5	20.4	20.0	−2
1.0	0.05	0.1	100	20	10	Standard = 20		
2.0	0.10	0.2	200	40	20	19.8	20.0	+1
3.0*	0.15	0.3	100	20	10	19.6	20.0	+2
4.0*	0.20	0.4	100	20	10	19.8	20.0	+1

* The solutions of the precipitate obtained from the 3 and 4 cc. samples were made up to 6 and 8 cc., respectively, and 2 cc. aliquots transferred to the 100 cc. flasks.

= 39. Theory 40. Error + 2.5 per cent. Reading obtained with 4 cc. of solution = 10.3. Theory 10.0. Error −3 per cent.

The amount of precipitate obtained and the development of the color are in direct proportion to the amount of P present in the sample in quantities varying from 0.0125 to 0.2 mg. of P. This is shown in Table I.

Trichloroacetic acid in the concentrations used does not interfere with the precipitation and determination of the phosphorus present.

The presence of Na, K, Ca, and Mg in concentrations comparable to those found in serum and also the following organic com-

pounds do not interfere with the precipitation and determination of the phosphorus: Dextrose 300 mg. per 100 cc., urea 300 mg.

TABLE II.

Recovery of Phosphorus Added to Serum.

Serum.	Inorganic P present.	P added.	Total inorganic P found.	Total inorganic P present.	Error.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
10	0.078	0.0125	0.088	0.090	−2.2
10	0.078	0.025	0.102	0.103	−1.0
10	0.078	0.050	0.127	0.128	−0.8
21	0.050	0.0125	0.062	0.062	±0.0
21	0.050	0.025	0.073	0.075	−2.6
21	0.050	0.050	0.105	0.100	+5.0

TABLE III.

Inorganic Phosphorus Content of Normal Adult Serum (Twenty-Two Consecutive Determinations).

Serum.	Inorganic P per 100 cc.	Serum.	Inorganic P per 100 cc.
	mg.		*mg.*
30	3.7	41	3.2
31	4.0	42	3.9
32	3.7	43	3.9
33	3.5	44	3.7
34	3.7	45	3.8
35	3.7	46	3.7
36	3.6	47	4.3
37	3.5	48	4.0
38	4.2	49	3.6
39	3.7	50	3.7
40	4.0	51	3.8
		52	3.7

per 100 cc., uric acid 10 mg. per 100 cc., creatinine 20 mg. per 100 cc. (present as creatinine zinc chloride), creatine 5 mg. per 100 cc., and acetoacetic acid 100 mg. per 100 cc.

The results given in Table II indicate that known amounts of phosphorus added to serum, the inorganic phosphorus content of which has been previously determined, may be quantitatively recovered.

Table III gives the results of the determination of the inorganic phosphorus in the sera of normal adults. The ages ranged from 20 to 35 years. The blood was removed between 9 and 11 a.m. and the serum separated within $1\frac{1}{2}$ hours. The determinations were performed within 36 hours of the time the blood was obtained.

Table IV gives the results of the determination of the inorganic phosphorus in sera of infants who showed no clinical evidences of rickets.

TABLE IV.

Inorganic Phosphorus Content of Normal Infant Serum.

Serum.	Age.	Inorganic P per 100 cc.
	mos.	*mg.*
10	12	5.2
19	13	4.6
20	14	5.8
21	16	5.0
22	10	5.3
53	4	5.7
55	36	6.4
Average		5.4

DISCUSSION.

The use of a solution of strychnine molybdate for the precipitation of phosphorus was first used by Pouget and Chouchak (1). The reagent as devised by these authors had to be used immediately after its preparation. Subsequently, Kober and Egerer (2) modified and improved the reagent so that it "was stable and gave quantitative and constant results." Other modifications have been made by Bloor (3), Medinger (4), Kleinmann (5), and Embden (6).

The reduction of molybdic acid with the production of a blue color has been known for many years. Some time ago, Taylor and Miller (7) devised a method for the determination of small amounts of phosphorus which depended on the precipitation of ammonium phosphomolybdate with the subsequent determination of the amount of molybdenum present by its reduction with phenylhydrazine. Quite recently a method has been devised by Bell and Doisy (8) which depends on the selective reduction of

the molybdenum present as phosphomolybdic acid, in an excess of molybdic acid, with hydroquinone as the reducing agent. The present method depends on the precipitation of the phosphorus as strychnine phosphomolybdate, with the strychnine molybdate reagent as devised by Embden, and the reduction of the molybdenum present in the precipitate by means of potassium ferrocyanide and HCl.

The precipitation of the phosphorus takes place very rapidly. 5 minutes after the addition of the strychnine molybdate reagent over 95 per cent of the inorganic phosphorus present is precipitated, and in 10 minutes the precipitation is complete, as no increase is obtained if the mixture is allowed to stand for ½ hour. The precipitate is slightly soluble in water, and if too much water is used for washing, erroneous results will be obtained. When the quantity of water indicated in this method is used it will be found that the amount of precipitate in solution in the second washing is so slight that it produces no appreciable error. Also the amount of the reagent left after the second washing is so small that if it is placed in a 100 cc. flask and potassium ferrocyanide and HCl added no perceptible green color can be observed. It is not necessary for the precipitate to be mixed with the added water to produce thorough washing.

The green color produced by the reduction of the molybdate is very stable and no special precautions are needed when it is being read in the colorimeter. If the solutions are allowed to stand a long time it will be found that the color tends to become more intense and of a bluish tint, this change taking place more rapidly in the weak solutions than in the strong ones. If a weak solution is read at 40 in the colorimeter with the standard at 20, in 3 hours time it will change to about 39 producing an error of +2.5 per cent, while a strong solution will change over night from 10.5 to about 11.0 producing an error of −5 per cent. No error will be obtained from this source if the reading is made during the first hour after the addition of the potassium ferrocyanide and HCl.

The amount of potassium ferrocyanide added may be varied slightly without effecting the result. The amount added, however, should be between 19 and 21 cc. The HCl should be added from a burette as a variation in the amount added effects the rapidity with which the color develops. The amount added

should be between 9.8 and 10.2 cc. It is also necessary that the volume of fluid in the known and unknown during the development of the color should be approximately equal.

It is of interest to observe the concentration of the inorganic phosphorus in normal human serum, as determined by various other methods. Greenwald (9) found the "acid-soluble" phosphorus to vary from 2 to 6 mg. of P per 100 cc. Marriott and Haessler (10) found from 1 to 3.5 mg. of inorganic phosphorus per 100 cc. and Bloor (11) 1.9 to 3.8 mg. of inorganic phosphorus per 100 cc. of normal adult serum. Feigl (12) found the "acid-soluble" phosphorus to be under 4 mg. per 100 cc. Two determinations of serum from normal adults are recorded by Bell and Doisy (8) who found 3.5 and 3.9 mg. of inorganic phosphorus per 100 cc. Howland and Kramer (13) report an average of 2.1 mg. of inorganic phosphorus per 100 cc. in the serum of normal adults and an average of 5.4 mg. in the serum of twelve non-rachitic infants. The presence of a high inorganic phosphorus content in the serum of non-rachitic infants reported in the present paper is in agreement with the high content, first recorded by these authors.

The inorganic phosphorus content of normal adult serum as determined by the present method is slightly higher than recorded by most observers. Since it is possible that some of the "unknown phosphoric acid combinations" as well as the inorganic phosphorus might be extracted with the trichloroacetic acid, duplicate determinations were performed using trichloroacetic acid and acid ammonium sulfate as the extracting agents. Identical results were obtained. The possible extraction of some of the "unknown phosphoric acid combinations" by the strongly acid strychnine molybdate reagent was also considered. The supernatant fluid after its separation from the precipitate was allowed to stand 24 to 48 hours. A definite precipitate was found in the fluid from the serum while a barely perceptible amount was present in the fluid from the standard. It is highly improbable that this definite precipitate would have formed in the fluid from the serum if the "undetermined phosphoric acid" had been previously extracted.

The method as described above was devised by the author for the study of the inorganic phosphorus content of the serum in various clinical and experimental conditions. The principle of the

method, however, is applicable to the determination of the acid-soluble and lipoid phosphates but as the experiments being undertaken at the present time include the estimation of only the inorganic phosphorus content of serum, the details for the determination of the acid-soluble and lipoid phosphates have not been worked out.

CONCLUSIONS.

1. A rapid colorimeter method for the determination of the inorganic phosphorus in 1 cc. of serum has been described.

2. The presence of Na, K, Ca, Mg, dextrose, urea, uric acid, acetoacetic acid, creatinine, and creatine in the concentrations found in normal and pathological sera does not interfere with the determination of phosphorus by the technique described.

3. The results obtained on serum are accurate to within $\pm$ 5 per cent of the amount of inorganic phosphorus actually present.

4. Known amounts of phosphorus (as KH_2PO_4) added to serum may be recovered quantitatively.

5. The inorganic phosphorus content of the serum of normal adults is singularly constant, the variation in nineteen out of twenty-two cases being only from 3.5 to 4.0 mg. per 100 cc. The serum of normal infants has a much higher inorganic phosphorus content than is present in normal adults.

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9. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.
10. Marriott, W. McK., and Haessler, F. H., *J. Biol. Chem.*, 1917, xxxii, 241.
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13. Howland, J., and Kramer, B., *Am. J. Dis. Child.*, 1921, xxii, 105.

VITAMINE STUDIES.

IX. THE INFLUENCE OF THE DIET OF THE COW UPON THE QUANTITY OF VITAMINES A AND B IN THE MILK.*

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The suggestion that Funk[1] offered in 1913 that there was probably a definite relationship between the amount of vitamine secreted in the milk and that ingested in the food has become a well established fact. Since then many investigators have referred to this relationship, but it is only recently that any have carried out experiments with laboratory animals to prove it definitely. Without attempting to review completely the work done in this field attention may be called to a few important investigations.

In 1916, McCollum, Simmonds, and Pitz[2] stated that the two factors, vitamines A and B, pass into the milk only as they are present in the diet of the mother. These investigators observed the ability of the female rat to rear its young when confined to rations known to be adequate for growth when sufficient amounts of vitamines A and B are added, and inadequate for growth when either of these factors is omitted. They had no means of determining whether the omission of either vitamine A or B affected the amount of milk secreted by the rat and, therefore, whether the young received enough milk to permit normal growth. In

* Published with the approval of the Director as Paper No. 285, Journal Series, Minnesota Agricultural Experiment Station.

[1] Funk, C., *Biochem. J.*, 1913, vii, 211.

[2] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33.

1918, Steenbock, Boutwell, and Kent[3] noting the variations in the amount of vitamine present in butter fat surmised that perhaps the rations on which the butter fat had been produced might be at fault. However, they further state that the ration is not the only factor to be considered as the butter fat produced by a cow fed exclusively on alfalfa hay was found to contain no demonstrable amounts of vitamine. They do not comment upon the fact that the butter had been kept in an unsalted condition in a poorly iced refrigerator for about 3 weeks and that mold had developed upon the surface; nor do they make any statement in regard to the quality of the alfalfa used. In 1920, Hess and Unger[4] suggested that an insufficiency of vitamine A in milk might be due to the winter fodder of the cow.

In 1919, Barnes and Hume[5] and Dutcher, Pierson, and Biester[6] announced simultaneously that they had noticed seasonal variations in the antiscorbutic properties of cow's milk. Experimental proof of this variation was later published by Hart, Steenbock, and Ellis,[7] Dutcher and coworkers,[8] and Hess, Unger, and Supplee.[9] Inasmuch as each of these investigators used a somewhat different method for studying this variation, the conclusion seems justified that the vitamine C content of cow's milk can be influenced materially by the food of the cow. However, Hughes, Fitch, and Cave[10] have recently reported that the milks of cows fed a vitamine-rich ration and a vitamine-poor ration showed no difference as to vitamine C, but that both vitamines A and B were decidedly greater in the milk from a vitamine-rich ration. The same was true of the butter made from the milk produced on these two rations.

[3] Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.

[4] Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.

[5] Barnes, R. E., and Hume, E. M., *Biochem. J.*, 1919, xiii, 306.

[6] Dutcher, R. A., Pierson, E. M., and Biester, A., *Science*, 1919, l, 184.

[7] Hart, E. B., Steenbock, H., and Ellis, N. R., *J. Biol. Chem.*, 1920, xlii, 383.

[8] Dutcher, R. A., Eckles, C. H., Dahle, C. D., Mead, S. W., and Schaefer, O. G., *J. Biol. Chem.*, 1920–21, xlv, 119.

[9] Hess, A. F., Unger, L. J., and Supplee, G. C., *J. Biol. Chem.*, 1920–21, xlv, 229.

[10] Hughes, J. S., Fitch, J. B., and Cave, H. W., *J. Biol. Chem.*, 1921, xlvi, p. 1.

Attention has already been called to the work carried on in this laboratory in regard to the variation in the vitamine C content of milk due to the food of the cow. The experiments which were being carried out at that time offered an excellent opportunity for the study of vitamines A and B of milk produced under favorable and unfavorable conditions. Up to this time only vitamine B, as it existed in the milk, had been studied, all investigations of vitamine A being made on the butter which had been separated from the milk.

The work of Hopkins and Osborne and Mendel, the most notable experiments in which milk is used as a source of vitamine B, will be discussed later on with the experiments which embody this paper.

EXPERIMENTAL.

The experiments described in this paper are the first, in so far as we are aware, in which both vitamine A and vitamine B of cow's milk have been quantitatively studied with respect to the changes which may occur in the milk due to a change from pasture to dry feed. In order to study such changes the amount of milk fed must necessarily be the minimum which would produce satisfactory growth. To find this minimum, experiments were begun in December, 1919, with the mixed milk from a Holstein and a Jersey cow which were on a vitamine-poor ration and whose milk was being used in another experiment.[8] Although at that time the actual details in carrying out the experiment differed somewhat from those which were finally adopted the results of these earlier experiments check very satisfactorily with those reported in this paper.

The plan first followed was that used by many investigators; *i.e.*, to start with a small amount of milk to furnish the vitamine which had been omitted from the ration and gradually to increase the milk until an amount was being given which just gave satisfactory results. 5 cc. were arbitrarily chosen as the minimum amount. This method of procedure was not suited for our work as the rats lost in weight very rapidly on the low vitamine ration and their condition became so precarious that they did not respond to small additions of the milk which was probably becoming poorer in vitamines each day. From these results it was decided that

10 cc. daily were the minimum amount of milk which would furnish adequate amounts of vitamines A and B, but in order to be certain of the outcome duplicate groups of rats were started which received 15 cc. of milk daily.

The milk used in these experiments was chosen to represent two types of milk: that produced by cows fed a ration presumably adequate in all respects, and that produced by cows fed a ration known to be deficient in vitamines but adequate in protein, mineral matter, and energy value. The ration fed the former group, which was the Station herd and includes Holstein, Jersey, Guernsey, and Ayrshire cows, was made up as follows:

	Parts.
Corn	200
Bran	200
Oats	200
Oil meal	140

Roughage was fed in the form of alfalfa hay and corn silage. During the summer months when the cows were on pasture this ration was continued but fed in smaller amounts than in the winter months. The ration which was fed to the latter group, which included two Holstein cows, consisted of equal parts of ground oats, ground barley, wheat middlings, and gluten feed with timothy hay and oat straw for roughage. This roughage was used for the first 2 months of the experiment, when the timothy hay was taken out as it was thought that it might be furnishing considerable amounts of vitamines to the ration. This ration is undoubtedly poor in vitamine A and furnishes inadequate amounts of vitamine B, and is a poorer ration than is fed during the winter months on the dairy farms producing the milk for large cities. During the period this ration was fed a fair flow of milk was maintained. The butter fat of the milk averaged 3.4 per cent which increased to 3.8 per cent after the cows had been on pasture for 3 weeks. These cows were placed on the vitamine-poor ration October 1, 1920, and continued on it until April 28, 1921, when the ration was changed to that of the Station herd. On May 16, 1921, the cows were turned out to pasture, the dry feed, however, being continued. The cows were milked morning and evening and samples of the mixed milk were taken for the feeding experiments.

The time for starting the rats on their experimental rations was so chosen that the milk used would be representative of a milk produced in midsummer when all conditions of feeding and pasture were good; in fall when the pasture was beginning to fail; in midwinter when the cows had to depend entirely on grain mixtures, silage, and hays; and in late winter and early spring when the effect of the change from winter feed to summer feed could be noted.

The rats used in this experiment were healthy, normal rats selected from our breeding colony. As far as possible five or six rats weighing from 65 to 75 gm. each were selected for each group. These were kept in separate cages so that a record could be kept of their food intake. The usual laboratory precautions were taken in regard to the sanitary conditions of the cages, drinking bottles, and feeding dishes. The rats themselves were kept free of animal parasites by the application of pine oil.[11]

The milk in 10 or 15 cc. portions, as the conditions of the experiment called for, was given to each rat the first thing in the morning and after the first few weeks of the experiment had passed the milk was consumed within a few hours, at least before it became sour. A little difficulty was experienced at first in getting the young rats to drink all of their milk but this difficulty did not extend over a sufficiently long period to affect the results of the experiment.

The basal ration used in our experiments was as free from vitamines as it is possible to make such a ration under the present conditions of our knowledge as to the nature of vitamines. The casein, which would be the most objectionable constituent in a vitamine-free ration because of adsorbed impurities, was made according to a method which is used in this laboratory and which we believe gives a very pure product.[12] The preparation of vitamine B might carry small amounts of vitamine A. However, inasmuch as the wheat embryo, used as a source of vitamine B, was first thoroughly extracted with ether, the amount of this vitamine in the subsequent alcoholic extract would doubtless be very small as it has been reported[13] that ether removes vitamine

[11] Kennedy, C., *Science*, 1921, liii, 364.

[12] Palmer, L. S., and Kennedy, C., *J. Biol. Chem.*, 1921, xlvi, 571.

[13] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549. Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xlii, 131.

The rats which were fed the milk produced in the fall months are included in this group due to the fact that for the first 2 months of the feeding period the cows were still on pasture. The fall milk was from the Station herd. There is no outstanding difference in the fall milk curves, Chart II, and the summer milk curves.

Chart II. Fall Milk.

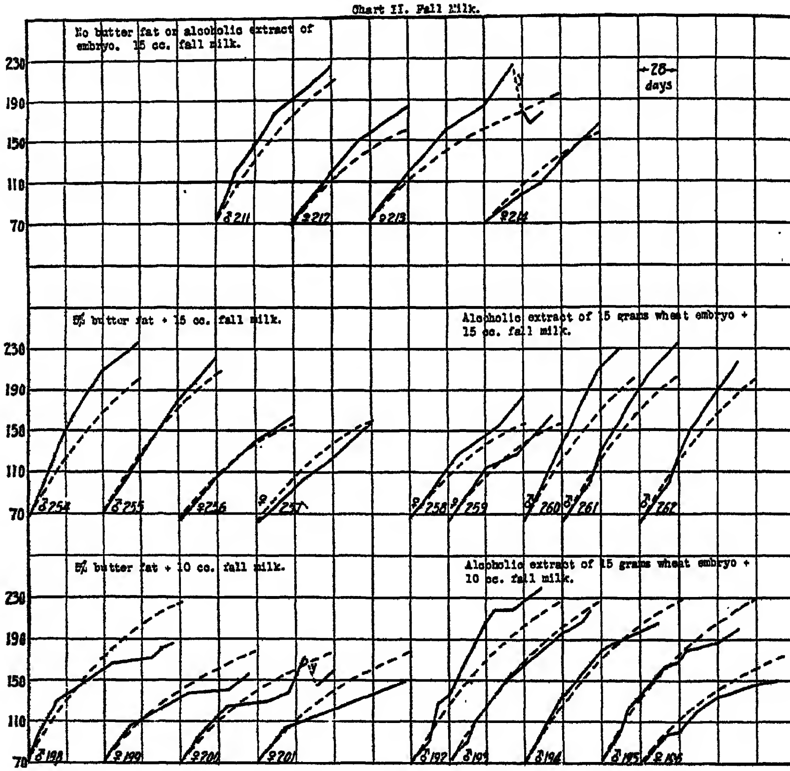

CHART II. Rats 192 to 201 inclusive, Rats 254 to 262 inclusive, and Rats 211 to 214 inclusive were placed on the experimental ration Sept. 1, 1920. The milk used for these rats was supplied by the Experiment Station herd. During September and October the pastures were green and the grass abundant. The first hard frost came Nov. 2 when the cows were removed from the pasture. The curves for Rats 198 to 201 inclusive show that the amount of vitamine B in 10 cc. of this milk is inadequate to support normal growth after 6 to 8 weeks. Comparison of these curves with those of Rats 304 to 307 inclusive (Chart VI) shows that the failure to make normal growth is not due to the fact that 10 cc. of milk will not furnish enough vitamine B but that 10 cc. of milk from cows feeding on poor pasture is not adequate.

The summer milk curves fall away from the normal from the effects of drying of pasture, and the fall milk curves start to fall off when the pasture season ends.

Group II, Charts III, IV, and V.—This group includes the rats fed the winter milk produced by the two Holstein cows. These cows had been placed on the vitamine-poor ration in October, 1920, and the feeding of this milk to the rats began in December. This afforded 2 months in which the cows could get rid of the vitamines in their tissues. However, it could not be determined whether the milk was becoming poorer each day in vitamines and would finally become so depleted that it would no longer cause

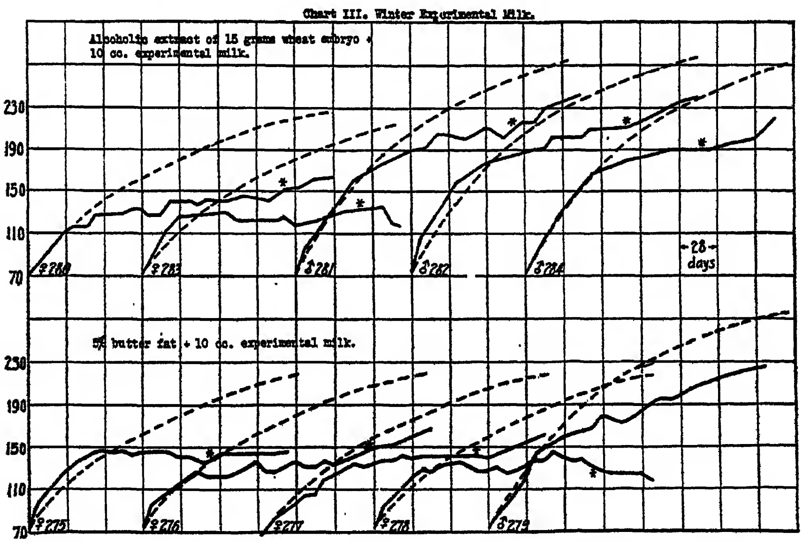

CHART III. Rats 275 to 284 inclusive were placed on the experimental ration Nov. 30, 1920. The milk used for these rats was supplied by the two Holstein cows which had been on vitamine-poor rations since the first of October. 10 cc. of this milk do not furnish adequate amounts of either vitamine A or vitamine B. Rats 275 to 279 inclusive became very thin and nervous during the course of the experiment and two rats, Nos. 275 and 278, died of lung infection near the end of the experiment. Although Rats 280 to 284 inclusive suffered severely in their growth from a lack of vitamine A, none showed signs of eye trouble indicative of a deficiency of this vitamine. May 1, 1921, the ration of the two experimental cows was changed to the herd ration and on May 16 the cows were turned out to pasture. This point is indicated by an asterisk on each curve.

Chart IV. Winter Experimental Milk.

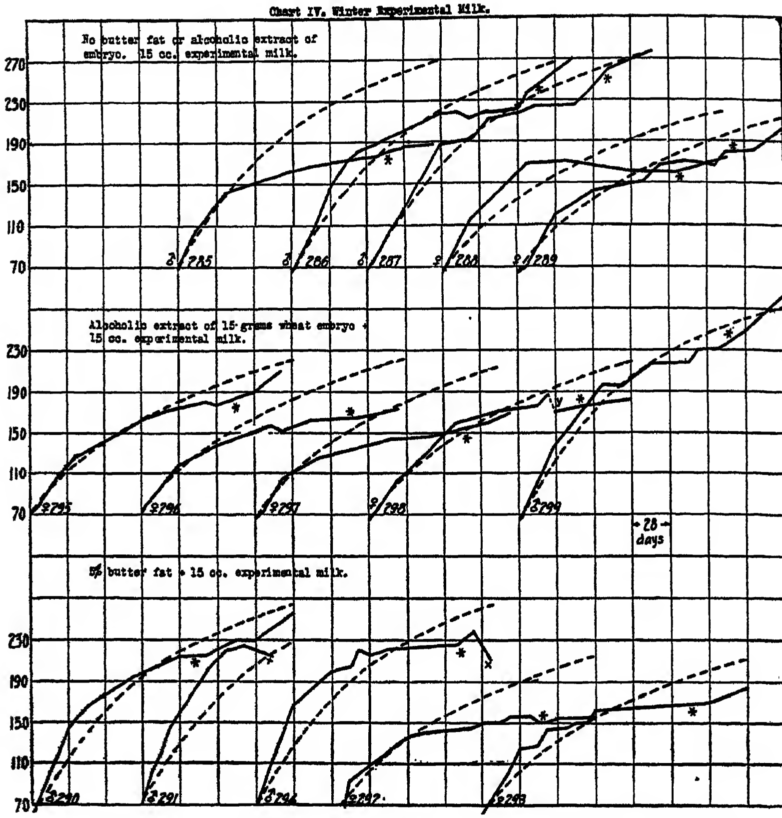

Chart IV. Rats 285 to 299 inclusive were placed on the experimental ration at the same time as the rats in Chart III; the only difference in their ration being that they received 15 cc. of milk instead of 10 cc. The increased amount of milk had a decidedly beneficial effect on the rats' growth curves. Although the increase in weight was normal or better than normal for a considerably longer period there was quite a long period when the rats were only able to maintain their weights. A comparison of this chart with Chart VI shows the great superiority of the quality of vitamine A contained in 10 cc. of winter milk from cows fed on an adequate ration over that contained in 15 cc. of milk from cows fed a ration known to be deficient in vitamines. The curves for Rats 285 to 289 inclusive show a greater divergence from the normal than do the other curves, but this is to be expected as there are two deficient factors in the ration of these rats while in the ration of the other rats there is but one deficient factor. Rats 291 and 294 died from an unknown cause. The cows were turned out to pasture at the point marked by the asterisk.

growth, or whether it was as deficient at this time as it ever would be, but still contained enough vitamine to cause some growth. 10 cc. of this milk do not furnish adequate amounts of either vitamines A or B after the first few weeks of feeding. The weights of the rats which received vitamine A and those which received vitamine B from 10 cc. of this milk began to fall away from the normal at about the same time although there is a slight extension of time in the case of the rats which received vitamine A from the

Chart V. Late Winter and Early Spring Experimental Milk.

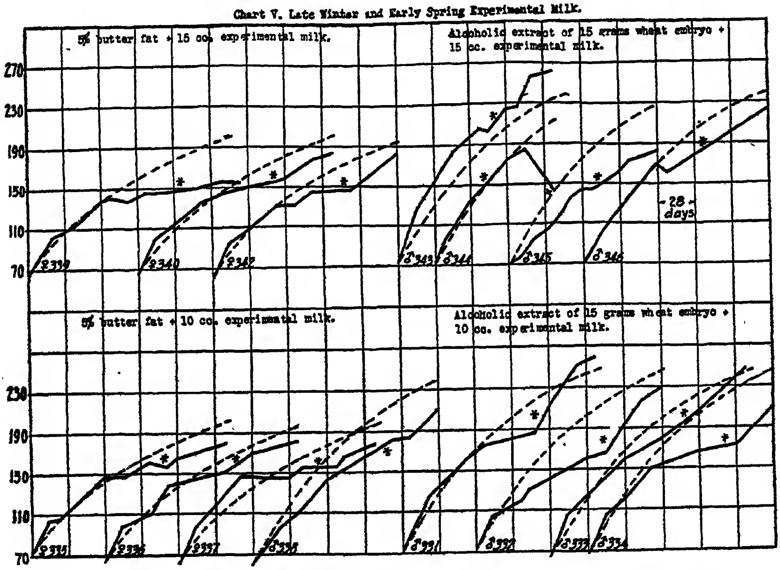

Chart V. Rats 331 to 346 inclusive were placed on the experimental ration Feb. 1, 1921. This group was started at this time to ascertain if the milk of the cows became progressively poorer in vitamines A and B as the length of time of feeding a vitamine-poor ration increased. Comparing this chart with Chart III, it is seen, in each group, that the rats stopped gaining weight at approximately 6 to 8 weeks after the beginning of the experiment. The curves in this chart (Chart V) appear better than those of Chart III because the rats were not on the experimental ration for as long a period, and, therefore, do not become as depleted. They also show a greater improvement after the cow's ration improves. These curves indicate, though they do not demonstrate, that if the vitamines are stored in the body of the cow it is only for a short time. The curves of the group receiving 15 cc. are only slightly better than those receiving 10 cc. Rat 344 died from an unknown cause.

milk. It is interesting to note that the male rats withstood the vitamine deficiency much better than the female rats. During the periods of the experiment although the rats which received vitamine A from 10 cc. of milk became thin, they had no xerophthalmia.

Further proof of the paucity of vitamine A in this milk is shown in Chart IX. The rats whose curves appear in this chart obtained their supply of vitamine A from butter that had been made from cream separated from this milk, during the last 4 weeks of the experiment, when the milk was undoubtedly very poor in vitamines. The butter was placed in small containers under an atmosphere of CO_2 and stored in an ordinary refrigerator, the temperature of which was approximately 10°C. Two groups of rats were fed purified rations carrying in one case 5 per cent of this butter

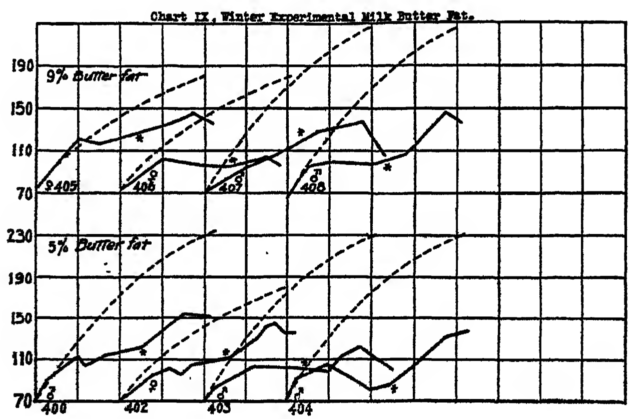

Chart IX. Rats 400 to 408 inclusive were placed on the experimental ration in the winter of 1921. The basal ration carried the alcoholic extract of 15 gm. of wheat embryo and in the case of Rats 400 to 404 inclusive, 5 per cent of butter fat made from the vitamine-poor milk, and in the case of Rats 405 to 408 inclusive, 9 per cent of the same butter fat. The asterisk indicates the point at which the fat in the ration of Rats 400 to 404 inclusive was increased to 15 per cent, and in the ration of Rats 405 to 408 inclusive to 20 per cent. That the rats did not improve very materially in this last group seemed to be due to the fact that the rats did not eat well of the ration because of its excessively greasy nature. Improvement in the former group took place on increase of the fat from 5 to 15 per cent but the curves never coincided with the normal. In all of our experimental rations 5 per cent of good butter fat has carried sufficient amounts of vitamine A for normal growth.

fat and in the other case 9 per cent. When the rats had ceased to grow the butter fat in each ration was increased to 15 and 20 per cent respectively. This increase gave only slightly better results. We believe that this deficiency is due to no other cause than to a lack of vitamine A in the ration of the cows as extreme care was exercised both in the separation and churning of the cream and in the subsequent storage of the butter.

The rats receiving their vitamine B from 10 cc. of milk became very nervous and timid and two, Rats 275 and 278, died of lung infections. In other groups where there was a deficiency of this vitamine the same lung infection occurred. None of the rats in this group showed a quick or marked improvement in their weights after the cows had gone to pasture. Whether these rats were in too depleted a condition to improve after the cows had gone to pasture, or whether the cows had become so depleted in vitamines A and B that 6 weeks was not a long enough period to show an improvement in the vitamine content of their milk, could not be definitely determined, although the rats of Chart V which had received the experimental milk for a shorter period and were in better physical condition showed a more decided upward trend in their curves. Perhaps the most striking inference which can be drawn from a comparison of Charts III and IV with Chart V is in connection with the observation that the rats on comparable rations grew to about the same weight before the vitamine deficiency became evident. As pointed out previously, there is no evidence to indicate that the milk fed to the rats of Chart V was any poorer in vitamine at the beginning of the experiment than that received by the rats in Charts III and IV, although the experiments labeled "Winter Experimental Milk" began 2 months before those designated "Late Winter and Early Spring Experimental Milk." As a matter of fact, the indications are rather that the quantitative deficiencies of the milk with respect to the vitamines were no greater in the late winter than several months earlier. The inference which we can draw from these data is either that the actual weight of vitamines required for growing rats of 70 to 150 gm. weight, a period of very active growth, is less than in larger rats, or that a certain period has been reached in the life cycle of the rat when the demand for vitamine is greatest. That this period is that of adolescence is indicated by the fact that

the rats in the experiment began to show signs of vitamine deficiency at the age when this species of animal attains sexual maturity.

The rats which received 15 cc. of this same winter milk maintained a normal growth curve for a considerably longer period than those which received 10 cc. but there was a long period when the rats were only able to maintain their weights.

Group III, Charts VI and VII.—The milk used for this group was the winter milk from the Station herd. The rats receiving 10 cc. of milk daily made remarkably good growth; in fact, it equalled that of those receiving 15 cc. This is of interest in view of the fact that Osborne and Mendel[15] have reported that 16 cc. of whole summer milk were necessary for satisfactory growth with a ration that was supplying the vitamine A. A comparison of Chart VI with Chart IV shows the great superiority of the quality of vitamine A contained in 10 cc. of the Station herd winter milk over that contained in 15 cc. of the winter milk from the two experimental cows. 15 cc. of the latter milk contained 0.526 gm. of butter fat and were much less efficient for growth than 10 cc. of the former milk which contained only 0.392 gm. of butter fat.

From the excellent growth of these rats we can conclude that a winter ration for cows may be made perfectly satisfactory as far as vitamines A and B of the milk are concerned by the proper combination of grain and leafy foods.

It will be noted that throughout the experiment there were only a few of the rats that reproduced. While it would have been desirable to have had reproduction, it was not the aim of the experiment to find a quantity of milk upon which perfect growth, as measured by the ability of the rat to reproduce at normal intervals would be attained, but rather to ascertain the effect of the cows fed on the quality and quantity of the vitamine produced in her milk. In order to obtain quantitative results in feeding it was, under the laboratory conditions at the time of the experiment, almost impossible to allow the rats to be together for more than a very short time each day and it is very difficult to obtain normal reproduction under such conditions. There were five females, however, which gave birth to young and reared them successfully.

[15] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537.

Attention may be called to the work of Hopkins[16] on the stimulating effect on growth of 2 cc. of cow's milk as an addendum to an artificial ration consisting of casein, fats, starch, sugar, and inorganic salts; and to his later work in which he substantiates this result. Because of these results it seemed necessary, after some of the preliminary experiments of this investigation had been completed and it was indicated that young rats could not grow on

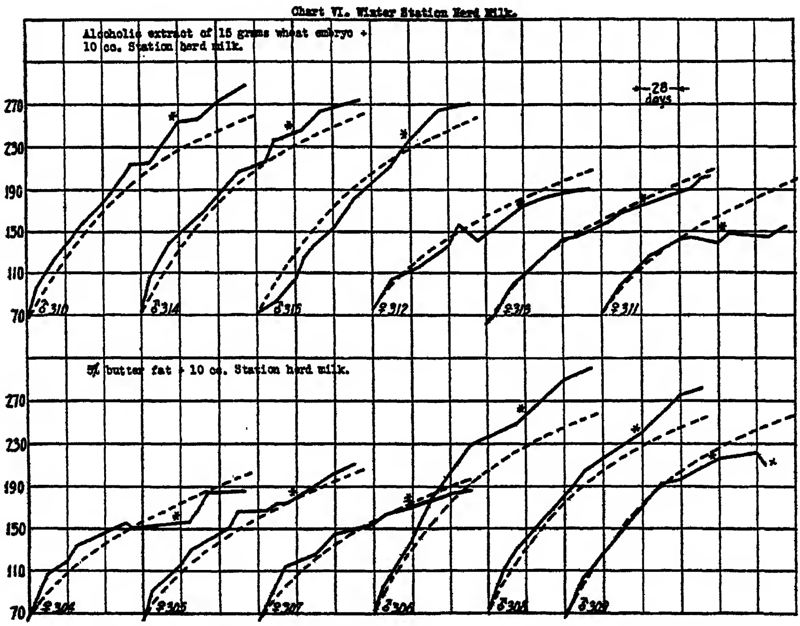

Chart VI. Rats 304 to 315 inclusive were started on the experimental ration Jan. 12, 1921. The milk fed to this group was furnished by the same herd that furnished the milk for the rats in Chart II. The rats receiving 10 cc. made remarkably good growth, in fact they equalled the growth of those receiving 15 cc. This fact demonstrates the value of an adequate combination of grain and leafy foods during the winter season. Rat 309 died of pneumonia. The time that the cows were turned out to pasture is indicated on the chart by an asterisk.

5 cc. of milk produced on a vitamine-poor ration, to further substantiate this result by repeating the experiment but to use a summer milk known to produce satisfactory growth when fed at

[16] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

a higher level. Therefore, two groups of rats were used: one group received, in addition to a ration which was complete in all respects except for its vitamine A, 5 cc. of milk to supply this vitamine; and a second group received, in addition to a ration which was complete in all respects except for its vitamine B, 5 cc. of the milk to supply this vitamine. The growth curves for these rats found in Chart VIII demonstrate very conclusively that 5 cc.

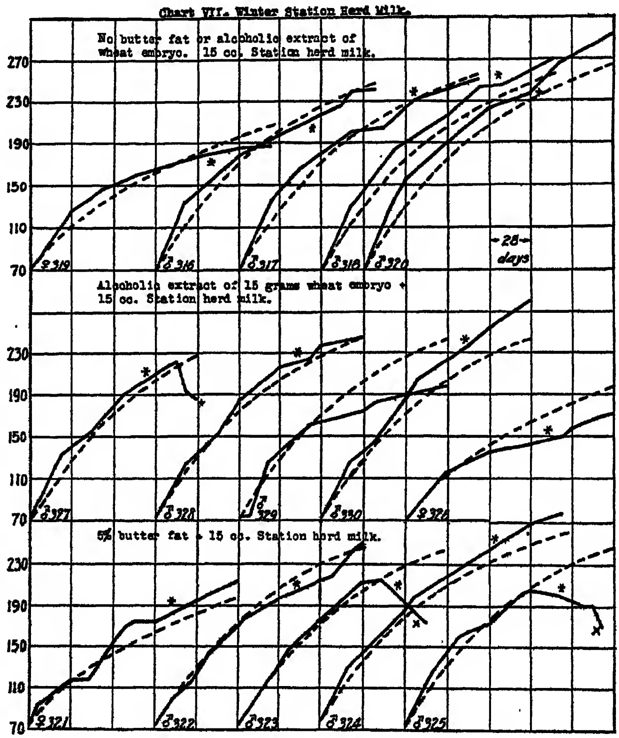

CHART VII. Rats 316 to 330 inclusive were started on the experimental ration Jan. 12, 1921, and received, daily, 15 cc. of milk from the Experiment Station herd. These rats evidently received sufficient vitamines from the start so that when the cows went into pasture any increase in the amount in their milk had little or no influence on the growth of the rats. That 15 cc. is ample to supply both vitamines A and B is shown by the curves for Rats 316 to 320 inclusive. Three rats, Nos. 323, 325, and 327, died from an unknown cause. The cows were turned out to pasture at the point marked by an asterisk.

of good quality summer milk will not promote normal growth in young rats. It is possible that the ration of the cows from which Hopkins obtained the milk he used was much richer in vitamines than that of the cows furnishing the milk for our experiment. That it is entirely a question of the ration of the cows seems to be clearly shown by the various results published by the different investigators. Hopkins has described carefully planned experiments in which he obtained satisfactory growth on a remarkably small amount of milk added to a purified ration. Osborne and Mendel[15] have described equally carefully planned experiments in which they state that "not until at least 16 cc. of fresh milk per

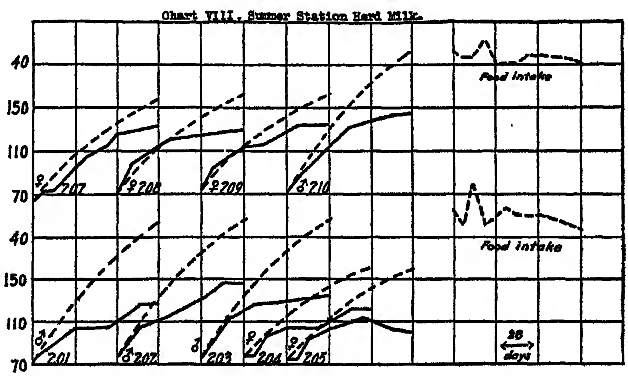

CHART VIII. Rats 201 to 210 inclusive were placed on the experimental ration in the summer of 1920. The milk used for these rats was the same as that used for the rats whose growth curves appear in Chart I. The failure to grow after the first 4 weeks is the result of a deficiency in vitamines A and B, owing to the small amount of milk (5 cc.) used as a source of these vitamines.

day were supplied along with the food mixture, was anything approaching a normal rate of growth secured. Even this amount sometimes failed." Later these investigators repeated this work using summer milk because they thought that the inferior quality of the milk, as a source of vitamine B, might be due to the winter ration of the cows, their earlier work having been carried out during the winter season when the cows were deprived of green pasture and were stall fed. In addition to the pasture grass, the cows were fed night and morning a ration consisting of corn gluten and

wheat bran together with hay and corn-stalks. Failure to grow became evident in approximately 25 days when the rats received 10 cc. of this milk, unpasteurized, from the beginning of the experiment. 15 cc. of the same milk barely sufficed as a source of vitamine B.

These results of Osborne and Mendel indicate that milk is a poor source of vitamine B. In our experience with milk we find that 10 and even 15 cc. of milk are inadequate to furnish either vitamines A or B when the milk is produced by cows which are feeding on a ration in which these vitamines are deficient, but that 10 cc. of milk are amply sufficient to furnish either vitamine provided the milk is produced by cows feeding on a ration which is adequate as to its vitamine content. It would, therefore, seem that milk becomes a good source of vitamine B when, in addition to feeding in pasture, the cow is given a grain and hay mixture rich in vitamines. Access to open pasture will not assure a ration rich in vitamines unless the pasture is always fresh and green. The feed in the pasture varies with the climatic conditions and in order to secure a milk uniform as to its vitamine content, it is necessary to give a good dry feed throughout the year. The unsatisfactory results obtained by the use of 15 cc. of summer milk, which Osborne and Mendel report, may be due to the fact that the dry feed ration of their cows was vitamine-poor and that practically the only source of vitamine B was pasture grass, a variable source.

That milk, as a source of vitamine B, may compare favorably with vegetable sources of this vitamine is shown in results which we have obtained in other work in this laboratory. We have found that while the alcoholic extract of 10 gm. of wheat embryo for each 100 gm. of ration will produce normal growth, this amount is not always dependable; therefore, it has been our custom, for some time past, to use the extract of 15 gm. of embryo. This extract, dried on 100 gm. of ration complete in all other respects, gives no better growth curves than 10 cc. of our Station herd winter milk when used as a source of vitamine B. This amount of milk adds to the ration of a rat 1.26 gm. of milk solids, which would not greatly enhance a complete ration unless the presence of vitamine C in the milk exerts a beneficial effect or that the milk contains some other food accessory which we have not as yet rec-

Chart X. Average Food Intake Per Week Per Rat.

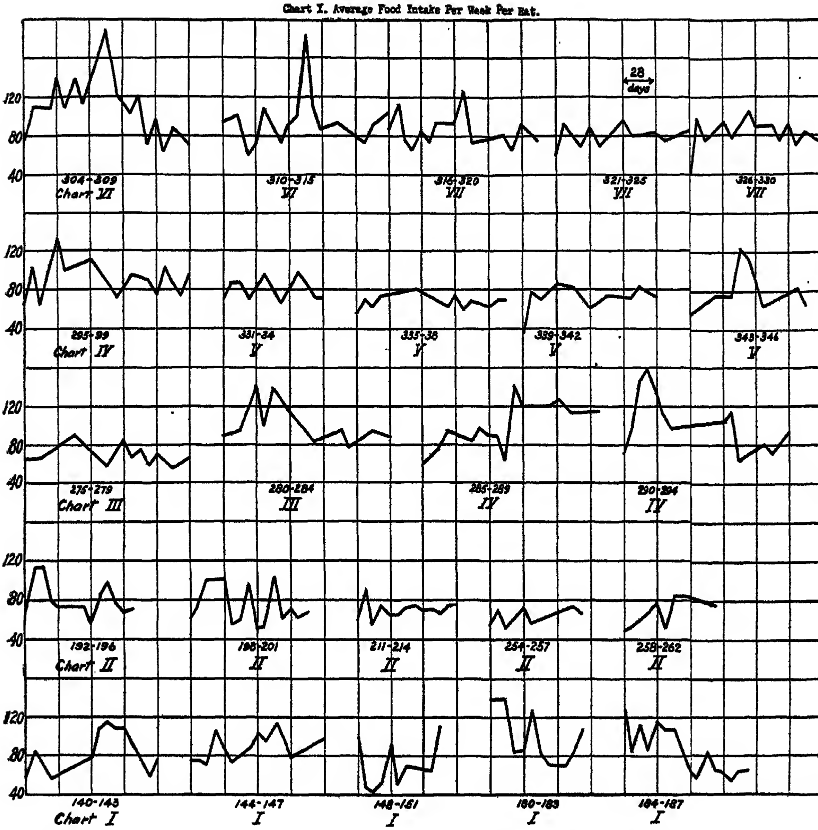

Chart X. The curves of this chart represent the average food intake per week per rat. Record of the dietary intake of each rat was kept throughout the experimental period and the average of the records comprising each group was used in computing these graphs. A comparison of the curves shows that the food intake is approximately the same, with slight variations, whether the animal is growing or merely maintaining its weight, which fact does not agree with the generally accepted theory that the food intake corresponds very closely with increase in growth. The food intake of the rats whose growth curves are found in Charts III, IV, and V was approximately the same during the first 6 to 8 weeks of the experiment, when growth was normal, as during the remaining period of the experiment when only maintenance of weight was accomplished. The actual food intake of the rats whose growth was excellent (Charts I, II, and VII) was no greater than that of the other groups (Charts III, IV, and V) which grew normally during only the first 6 to 8 weeks of the experiment and then merely maintained their weights. Moreover, the food intake for the rats of the former groups (Charts I, II, and VII) was less per gm. of body weight during the latter part of the experimental period than that of the rats of the latter groups (Charts III, IV, and V) during the same period of the experiment. It would seem, therefore, that the effect of the vitamine is not necessarily one of appetite stimulation but rather a stimulation of metabolic processes which promote growth.

The food intake for Rats 304 to 315 inclusive, Chart VI, does not parallel that of any of the other groups.

ognized. And again, baker's yeast which has recently come into great prominence as a rich source of vitamine B, has proved under the conditions of our experimentation, to be a less valuable source of vitamine B than we formerly supposed. We have found that when the yeast is mixed in the ration in the proportion of 10 gm. of yeast in each 100 gm. of ration that growth was not as good as when 10 cc. of our Station herd milk were used to supply the same vitamine. When the yeast was fed separately from the food mixture 0.6 gm. per day gave the same results as when the yeast was mixed in the ration in the proportion of 10 gm. to 100 gm. of ration. Osborne and Mendel[17] report that 15 cc. of summer milk are inferior to 0.2 gm. of brewer's yeast. We have found that the brewer's yeast, which we have been able to obtain, is much inferior in growth-promoting properties to baker's yeast.

SUMMARY.

Two types of milk, one produced on a ration typical of that used on some farms during the winter season and known to be deficient in its vitamine content, and a second representing that produced on a ration carrying ample amounts of vitamines A and B, have been used in this investigation. Each milk was fed so as to show in as nearly a quantitative manner as possible its content of vitamines A and B. Growth curves are given which show the possibility of growth on low and high levels of each milk. The importance of feeding the cow a ration adequate as to its vitamine content is demonstrated.

CONCLUSIONS.

1. The presence of vitamines A and B in cow's milk is entirely dependent upon their occurrence in the ration.

2. Stall fed cows will produce a milk rich in vitamines provided their ration consists of a proper combination of grains and leafy foods.

3. A vitamine-rich milk is not necessarily correlated with access to pasturage.

4. 10 cc. per day of either winter or summer milk is adequate to furnish either vitamine A or B to a rat provided the ration of the cow carries each in amounts adequate to meet her requirements.

[17] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 515.

5. 5 cc. of the same milk that produced normal growth when used on a higher level does not furnish enough of either vitamines A or B to meet the requirements of growing rats.

6. The effect of the vitamine is not necessarily one of appetite stimulation but rather a stimulation of metabolic processes which promote growth.

In conclusion we wish to acknowledge the assistance of Mr. John W. Wilbur, formerly with the Division of Dairy Husbandry, in keeping careful oversight of the experimental cows whose milk was used in this investigation and in making weekly butter fat determinations on the milk.

STUDIES ON THE ACETONURIA PRODUCED BY DIETS CONTAINING LARGE AMOUNTS OF FAT.*

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The excretion of the acetone bodies—acetone, acetoacetic acid, and β-hydroxybutyric acid—in conditions in which the organism is not utilizing carbohydrate either through a deficiency of foodstuff of this kind in the diet or through the inability of the organism to metabolize the food when supplied, as in diabetes mellitus, has attracted attention for many years, and a large amount of literature has collected on the subject. In two papers recently published, Shaffer (1921, *a*, *b*) has summarized this literature, and has suggested certain methods of studying the problem which are somewhat different from those which have been used before. In this paper are reported some experiments which were carried out along the lines suggested, and which appear to support the theses advanced in these two articles.

In his first paper Shaffer (1921, *a*) reported experiments on the oxidation of mixtures of acetoacetic acid and glucose by alkaline hydrogen peroxide which showed that if there were present in the mixture one molecule or more of glucose for each molecule of acetoacetic acid, the acid was oxidized under suitable conditions of temperature, alkalinity, etc., but that if the relative concentration of glucose was less than this, the oxidation of the keto-acid was not as complete. In the second paper (Shaffer, 1921, *b*)

* A preliminary report of the clinical side of the work discussed was read before the meeting of the New York State Medical Association in Brooklyn, May, 1921, by Floyd R. Wright; a portion of the work formed part of a thesis presented for partial fulfilment of the requirement for the degree of Doctor of Philosophy at Washington University, St. Louis, in June, 1921, by Roger S. Hubbard.

he studied the problem from the point of view of the metabolism of human subjects, and concluded that a reaction of a similar nature takes place in the body. Since the appearance of these two articles Woodyatt (1921) has published a paper in which the subject is discussed from the standpoint of the practical treatment of diabetes, and in which data are presented which support the conclusions stated above.

The theory which has been developed in these papers, and on which the following paper is based, is that acetoacetic acid itself is not easily burned in the body, but that it forms with glucose, or with degradation products of glucose and related substances, a compound which is easily burned. To compounds which give rise, in the progress of metabolism, to acetoacetic acid the name "ketogenic" is given, while the name "antiketogenic" has been applied to compounds which furnish glucose or other related compounds with which the acetone body combines. The ketogenic compounds contained in the diets are the fatty acids contained in the fats and the α-amino-acids, leucine, tyrosine, phenylalanine, and possibly histidine which forms a part of the proteins. There is probably a molecule of the acetone bodies derived from each molecule of these compounds contained in the diet.

The amounts and source of the antiketogenic compounds contained in the diet are more uncertain. Glucose and related sugars, as levulose, form one source of these substances, whether taken as the sugars themselves or as the more complex carbohydrates. Protein yields glucose when fed to the total diabetic in amounts which vary with the different kinds of the foodstuff, and some percentage of the protein should therefore be included with the carbohydrate in figuring the total antiketogenic intake. There is, too, considerable data which indicate that glycerol yields glucose under some conditions, and so fat, from which glycerol is produced by hydrolysis in the organism must also be considered as a possible source of antiketogenic compounds.

The question is even more complicated than this, because it is not certain what derivatives of the glucose-forming α-amino-acids and glycerol act as antiketogenic compounds. Shaffer (1921, *b*) has pointed out this difficulty clearly. He states:[1]

[1] Shaffer (1921, *b*), p. 458.

". . . . the two carbon residues from glycocoll and the three carbon residues from the other sugar-forming amino-acids may have direct and immediate antiketogenic (ketolytic) action without condensation to glucose, and the same may be true of glycerol."

To determine the border-line diet which should just produce an excretion of the acetone bodies Shaffer (1921, *b*) calculated the molecular equivalents of the ketogenic compounds from fat and protein, and of the antiketogenic compounds from carbohydrate, protein, and the glycerol residue of the fat to the extent of the glucose which could be derived from them. From the analysis of the data so obtained he concluded that a diet containing 10 per cent of the calories in the form of protein, 10 per cent as carbohydrate, and 80 per cent as fat represented approximately the border-line diet. He studied this diet in the light of data contained in the literature and obtained experimentally, and showed that the theory was confirmed by such results as were available.

In the experiments reported in this paper an attempt was made to study this diet described by Shaffer, and diets in which the relative amounts of carbohydrate and fat were somewhat varied. To obtain a method of graphic representation of the various diets in terms of their "ketogenic balance" the following plan was adopted. The excess antiketogenic material derivable from protein, that is, the amount of glucose which protein would yield greater than that needed to bring about oxidation of the ketogenic material from the same protein, was calculated from the data presented by Shaffer (1921, *b*). He showed from the analyses of the α-amino-acid content of ox muscle given by Lusk (1917)[2] and from the glucogenetic power of protein, that there are twice as many gram molecules of antiketogenic substance (glucose) as of ketogenic compounds which can be derived from a given weight of this protein. Glucose is derived from ox muscle at the rate of 58 gm. for each 100 gm. of the protein ingested (Woodyatt, 1921), and there will be 29 gm. of excess glucose for each 100 gm. of this protein fed. The amounts of glucose and of acetoacetic acid which can be derived from different proteins vary, and 25 gm. have been chosen as a convenient average figure to express the amount of glucose available for additional antiketogenic

[2] Lusk (1917), p. 77.

action from 100 gm. of protein. This calculation is similar to that suggested by Woodyatt (1921). To the excess glucose from protein was added the glucose taken in the diet, and the sum was multiplied by 1.5 (molecular weight of glucose = 180; molecular weight of stearic acid = 284; of palmitic acid = 256; of oleic acid = 282; average = 270; $\frac{270}{180} = 1.5$) to convert the result into terms of its fatty acid molecular equivalent. This product was divided by the fatty acid content of the diet (95 per cent of the fat) and the ratio was multiplied by 100 to give the resulting expression in the form of per cent.

This formula for expressing the "ketogenic balance" of any diet is expressed as follows:

$$100 \times \frac{1.5\ (\text{weight glucose} + 25 \text{ per cent weight protein})}{95 \text{ per cent weight fat}}$$

In preparing the charts in this paper the total carbohydrate content of the diet has been used instead of the glucose content. Such a substitution introduces an error, as the intake of starch in grams should be multiplied by 1.1 to give the correct amount of glucose to which it is equivalent, but the difference between the values is almost certainly within the limit of error, and the total carbohydrate content is more easily calculated from published tables.

Before proceeding to a study of the experiments, attention must be called to some of the limitations and advantages of the formula given above. In the first place it is based on an assumption which did not hold exactly for any of the diets studied. The formula assumes that all of the fat contained in the diets was fed in the form of glycerides of the higher fatty acids—palmitic, stearic, and oleic—and such a diet could not be fed for any considerable period. In one of the experiments an attempt was made to approximate such a composition for a few days, as will be described below, but for the most part butter and cream formed a large percentage of the fat ingested. These fats contain relatively large amounts (up to about 8 or 9 per cent) of their fatty acids in the form of compounds of comparatively low molecular weight, and, therefore, yield more ketogenic material per gram than do fats not derived from milk. In the formula a figure

lower than 1.5 should be used to convert antiketogenic compounds expressed as glucose into molecular equivalents of fatty acids when these fats are included in the diet. Butyric acid, for example, has a molecular weight of 88, and if tributyrin were the only fat fed the sum of the antiketogenic compounds expressed as glucose should be multiplied by 0.49 ($\frac{88}{180}$) to express the fraction in terms of relative molecular concentrations. However, this error will not change the numerical value of the expression by more than 5 per cent; the error introduced by figuring from the carbohydrate content of the diet was of the same order of magnitude, and the two should practically compensate for each other.

The second objection to the formula has been indicated already. It is impossible to be sure that the figure used to express the excess antiketogenic material from protein is correct. The value will vary for different proteins as their content of leucine, tyrosine, and phenylalanine varies, and will also vary because the glucogenic power of different proteins is different. The effect of this uncertainty upon the numerical expression is illustrated by the following figures. In a diet in which 10 per cent of the calories is fed as protein, 10 per cent as carbohydrate, and 80 per cent as fat the numerical value of the expression given above is 55 per cent. The values of similar expressions in which different figures express the excess glucose from protein would vary from 31 per cent if its antiketogenic power is neglected, to 71 per cent if its ketogenic power is neglected, and the glucose which can be derived from protein is figured at 60 per cent of the total weight. If protein contains both ketogenic and antiketogenic materials—and this is almost certainly the case—the different figures lie well within the limits of error with which such formulas can be applied to the study of actual diets.

In case the antiketogenic effect of protein depends, not on glucose, but on the two and three carbon atom residues derived from the sugar-forming α-amino-acids the values of the expression would be much higher than 71 per cent. In that case an α-amino-acid would figure three times as efficient as glucose if a two carbon atom residue takes part in the reaction as an antiketogenic compound, or twice as efficient if a three carbon atom residue takes such a part. It seems almost certain that it would

be possible to detect such a marked effect as this experimentally, and an attempt has been made to interpret the data presented below in such a way as to solve this problem.

In the formula given no account has been taken of the possible antiketogenic effect of the glycerol radicle present in the fats. This radicle is probably the most uncertain of the different possible sources of antiketogenic compounds contained in the diet, and the part which it plays in the reaction can be better discussed after a study of the data obtained experimentally. If different diets are fed, and the degree of acetonuria is noted and compared with some such numerical expression of their ketogenic balance as that suggested, the value of the ratio at which the excretion of the acetone bodies becomes normal will represent the condition of ketogenic antiketogenic equilibrium. From the numerical value of this ratio it should be possible to determine whether the glycerol residue figures as a source of antiketogenic compounds. If this residue yields glucose in the organism, and this glucose acts as an antiketogenic compound, enough of such material would be furnished in each gram of fat to combine with one-sixth of the ketogenic material, so that only five-sixths of the total fatty acids will be free to combine with the antiketogenic compounds from carbohydrate and protein; in this case acetonuria should develop and clear up when the diet has a value of 83 per cent. If glycerol in fat does not produce antiketogenic compounds in the organism, all of the fatty acid will take part in the reaction with antiketogenic material from other foods, and the border-line diet will have a numerical value of 100 per cent. If glycerol figures as an antiketogenic compound in the form of a three carbon atom residue, one-third of the total fatty acid will combine with it, two-thirds of it must be burned by the help of other foodstuffs, and the border-line diet should have a value of 67 per cent. In the experiments reported here an attempt has been made to make such comparisons, and to determine whether the glycerol residue of fat does possess an antiketogenic action.

The study of diets which produce border-line acetonuria and at the same time maintain the body weight of the subjects is rather difficult. The diets are markedly different from those generally eaten, and many patients return a portion of the fat untouched. There is also a temptation to break the dietary

restrictions not unlike the temptation to which diabetics are subject, and it would not be necessary for a patient to ingest much more carbohydrate than is furnished to spoil an experiment. In the series presented here only such cases are included as could be studied under rather close supervision in a department devoted exclusively to the study of nutritional diseases. We wish here to express our thanks to Dr. S. T. Nicholson, Jr., the director of this department, and the dietitians and nurses attached to it for their cooperation in our experimental work.

The series included two experiments on a normal subject, one of which has been previously reported in another connection, and studies on four cases of arthritis who were undergoing the dietary treatment recommended by Pemberton (1917) in which the carbohydrate intake is reduced. These cases can probably be considered as normals for the purpose of such a study, although Pemberton and Foster (1920) have stated that such patients show a slightly increased concentration of sugar in the blood and an abnormal rise in blood sugar after the administration of large doses of glucose.

In all of the experiments the attempt was made to furnish enough food to each patient to maintain the body weight unchanged. To accomplish this the basal metabolism was determined with the Benedict portable respiration calorimeter (Benedict, 1918) and enough calories were fed to allow for the maintenance of basal equilibrium and for the probable activity of the patient. Usually the food provided for a bed patient was so calculated as to furnish 20 to 25 per cent more calories than his basal requirement called for, and this was found to be satisfactory for most of the subjects. It is desirable that the patients should be in nitrogen as well as in metabolic equilibrium, and at the same time that the protein content of the diet should be kept low to diminish the uncertain factor of its part in the ketogenic expression. In the experiments reported 10 per cent of the total calories were fed as protein in most of the diets studied. The relationship between the nitrogen intake and the output of nitrogen in the urine was determined, and it was found that there was little difference between them. If the excess antiketogenic compound had been figured from the urinary nitrogen instead of from the protein intake, the value of the ratio described would

not have varied beyond the limits of experimental error. The intake of carbohydrate and of fat formed, respectively, the sources of 10 and of 80 per cent of the calories in the basal diet, and of varying percentages—5 and 85 per cent, 15 and 75, 20 and 70 per cent—in the other diets studied. An attempt was made to feed each of these diets for a period long enough to determine the level of acetone excretion which corresponded to it, but it was usually necessary to change the more severe diets before such an equilibrium was established.

The diets used were figured from the tables given in Joslin's Diabetic Manual (Joslin, 1919); they were prepared under the direction of a competent dietitian, and food not eaten was weighed, and the proper allowance made in the record; a complete sample diet is given for one of the cases. While a majority of the patients ate the diets as furnished, two did not, and the results of the studies carried out on them are accordingly not wholly satisfactory. It has seemed best to include these cases in this report, however, as they serve as a check upon the results obtained upon other subjects.

The urines were sent to the laboratory daily. It was impossible to control the completeness of the collection through creatinine determinations because the presence of the acetone bodies in urine interferes with the method of analysis (Morris, 1915), and all of the cases except one showed a large excretion of acetone on all of the more severe diets fed. This lack of suitable control of the accuracy of collection made it seem best to record and plot the concentration of the acetone bodies as well as their total excretion. In some specimens marked variations in volume, total nitrogen content, and ammonia nitrogen content almost certainly show failure to collect accurate 24 hour specimens.

These daily urines were analyzed for acetone bodies by a method recently described (Hubbard, 1921) by which the acetone plus acetoacetic acid were determined together as acetone, and the β-hydroxybutyric acid was determined separately, also as acetone. Total nitrogen was determined by the direct Nesslerization method of Folin and Denis (1916), slightly modified to permit the use of the oxidizing and Nessler's reagents, described by Folin and Wu (1919). Ammonia determinations were made by the permutit method of Folin and Bell (1917). In some instances

other factors were studied which were connected more indirectly with the main problem. Total acidity was determined in the urine of two of the patients by the method described in Folin's Manual (Folin, 1916),[3] and its hydrogen ion concentration in one of the experiments by a colorimetric method using the standard universal buffer solution described by Acree, Mellon, Avery, and Slagel (1921). This solution was standardized before use against phosphate solutions of known hydrogen ion concentration. Besides these determinations on the urine the stripped weight of the patients was recorded, and in most instances the tension of carbon dioxide in the alveolar air was estimated. This determination was carried out in Cases II and III by the method of Marriott (1916) and in the other cases by the Fridericia method (Fridericia, 1914; Poulton, 1915).

The results obtained are given in Tables I to VI, and plotted in Charts 1 to 6. In these charts the diet is indicated at the top in terms of both total food and of percentage of the calories furnished by the three main classes of foodstuffs. When the diet varied much from day to day the average intake was made the basis of this plot. The numerical value of the expression

$$100 \times \frac{1.5 \text{ (weight carbohydrate)} + 25 \text{ per cent (weight protein)}}{95 \text{ per cent weight fat}} = \text{N per cent}$$

was plotted to correspond with the intake of food for each day, and the daily excretion of all the acetone bodies reckoned as the sum of the acetone which could be formed from them was plotted below it. Since slight increases of acetone above normal may be of considerable importance in this study, the plan was adopted, in two of the cases studied, of plotting the excretion of acetone bodies in terms of their concentration in the urine upon paper with logarithmic characteristics. This method shows differences which are actually slight but which may figure as large percentage increases because the normal amounts are small. In these plots the two fractions of the acetone bodies—acetone plus acetoacetic acid and β-hydroxybutyric acid—were plotted separately to show the relationship between the two fractions, and both were plotted as acetone to make the curves comparable. The concentrations

[3] Folin (1916), p. 103.

instead of the total excretions were used because of occasional failure in the collection of accurate 24 hour specimens.

Cases I and IV are reports of two experiments carried out on the same normal subject (one of the authors, R. S. H.). The data reported under the heading "Case I" have been previously presented (Hubbard, 1921) and are repeated here because a comparison with those obtained on the same subject in a later experiment show some things which are not as well brought out in other studies. The subject was a man 5 ft. 10½ in. tall, who weighed 165 lbs. and who at the time of the first experiment was 28 years old. During both of the periods he did light laboratory work while the experiments were going on. The first series of results was obtained before the appearance of Shaffer's papers in 1921 and the diet was differently planned from those used in the other experiments. The fat and carbohydrate were fed in different relative amounts—multiples of 50 gm., as a study of the table shows—and an attempt was made to feed sufficient protein to keep the caloric intake constant. The subject had been living on a normal mixed diet up to the first day of the experiment. There was a slight negative nitrogen balance during the first part of it, and a slight positive one after the diet had become more nearly normal, but the difference was not great enough in either case to affect the calculation of the probable ketogenic balance seriously. The development and clearing up of acetonuria is clearly shown in Table I and Chart 1. There was certainly an increased acetonuria on a diet which contained 250 gm. of fat, 50 gm. of carbohydrate, and 68 gm. of protein; this diet has a ketogenic balance of 42 per cent in terms of the formula suggested for expressing that balance. The excretion of the acetone bodies was slightly increased during the first 3 days of the experiment, but the increases were so slight that they cannot be attributed with certainty to the diet. The acetonuria completely cleared up when a diet having a ketogenic balance of 152 per cent was fed, and it seems certain that the border-line diet, that is, the diet representing ketogenic equilibrium, must lie between the two extreme diets fed, and probably does not lie far from that fed at the start of the experiment which has a ketogenic balance of 97 per cent. Attention should be called to the gradual increase and decrease of acetonuria as it developed and cleared up; a study of the table and chart makes it seem improbable that the second diet was fed long enough to cause a maximum excretion of acetone to correspond with its composition.

The experiment recorded under the heading "Case IV" was carried out on the same subject as was that recorded under "Case I." The height and weight were approximately the same as those given in the preceding paragraph, and the age was 33 years. The basal metabolism measured 1,750 calories per day. The diets were calculated and fed as here described, and the subject ate the entire amount of every diet provided. The collection of urine samples was accurate. There was some loss of weight during the first part of the experiment, but when diets were fed which caused an

TABLE I.

Case I.

Date.	Diet.							Urine.							
	Protein.		Fat.		Carbohydrate.		Calories.	Volume.	Total N.	NH_4N	Acetone + diacetic.		β-hydroxybutyric acid.	
1917	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
Mar. 12	96	15	201	69.5	100	15.5	2,573	935	12.5	0.748	0.7	0.006	2.2	0.020
" 13	96	15	201	69.5	100	15.5	2,573	1,100	17.0	0.748	4.4	0.048	4.4	0.042
" 14	96	15	201	69.5	100	15.5	2,573	905	16.6	0.776	7.2	0.065	3.5	0.032
" 15	68	10.1	251	82.4	50	7.5	2,711	1,095	17.8	0.787	34.2	0.374	18.8	0.206
" 16	68	10.1	251	82.4	50	7.5	2,711	815	14.6	0.815	58.0	0.473	69.0	0.562
" 17	68	10.1	251	82.4	50	7.5	2,711	1,180	12.5	0.914	68.0	0.802	112	1.42
" 18	72	11.7	175	63.7	150	24.6	2,448	980	13.4	0.964	28.0	0.274	35.9	0.352
" 19	72	11.7	175	63.7	150	24.6	2,448	1,040	11.5	0.849	10.3	0.107	5.8	0.061
" 20	72	11.7	175	63.7	150	24.6	2,448	860	9.75	0.670	3.3	0.026	3.4	0.029
" 21	72	11.7	175	63.7	150	24.6	2,448	810	10.6	0.667	2.8	0.023	3.4	0.026
" 22	72	11.7	175	63.7	150	24.6	2,448	610	9.94	0.490	1.4	0.024	4.0	0.024

Results of the determinations of the acetone bodies are expressed in terms of acetone.

excretion of only very small amounts of acetone the weight was quite constant. There was a slightly negative nitrogen balance as based on the determination of urine nitrogen during the first part of the period of study, and a slight retention later when the diet fed was more nearly normal. The excretion of acetone increased from 40 mg. on the day before the experiment to about 1.25 gm. after 4 days on a diet which contained 10 per cent

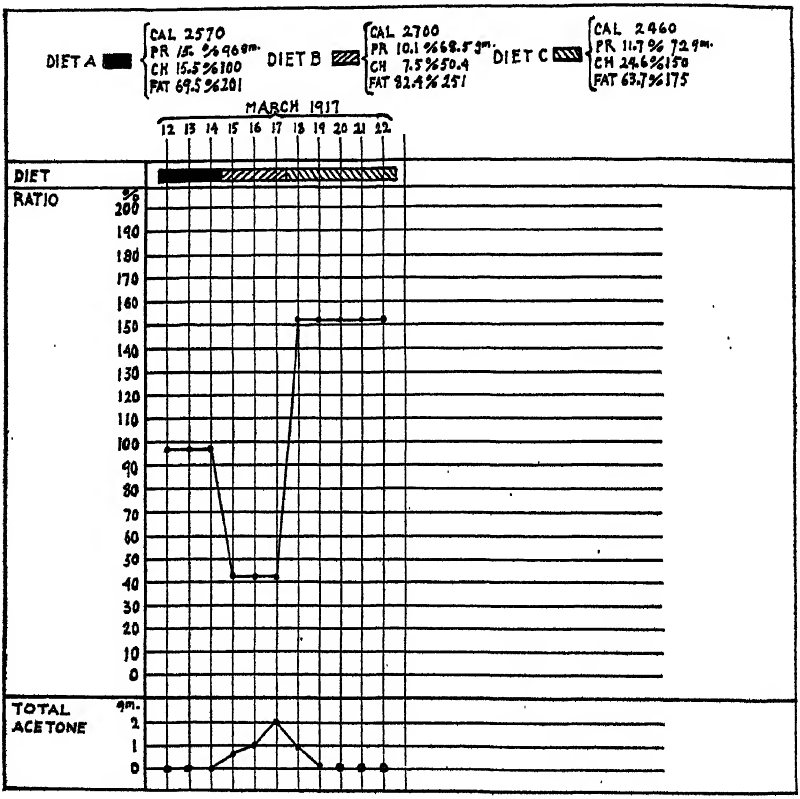


Chart 1.

of the calories as protein, 10 per cent as carbohydrate, and 80 per cent as fat; this increase makes it seem probable that there were more ketogenic than antiketogenic compounds in the diet. When the relative amount of carbohydrate in the diet was increased the excretion of the acetone bodies diminished, but did not return to the normal values when the diet contained 20 per cent of the calories as carbohydrate and 70 per cent as fat; oleomargarine and olive oil were substituted for the larger part of the butter fat in

this diet for 3 days, the 7th, 8th, and 9th of August, but the excretion of the acetone bodies was not measurably decreased further. However, the amounts of acetone excreted when this diet, which has a ketogenic balance of 108 per cent, was fed were not markedly different from those found on a less severe diet which had a ketogenic balance of 79 per cent, and were not very large in either case. The border-line of increased acetonuria appears to lie, for this case, between diets giving values of 78 and 108 per cent, although the value may be higher if the excretion of very small amounts of acetone is regarded as important in determining when ketogenic antiketogenic equilibrium has been established. It is noticeable, from comparing these two experiments on the same individual, that the excretion of acetone depends on the ratio between fat and carbohydrate rather than on the fat content of the diet.

Detailed Diet.

Case IV.

July 15.

Breakfast: Eggs, 2; Bacon, 20 gm.; Cream, 45 cc.; Butter, 15 gm.; Bread, 15 gm.; 10 per cent fruit, 70 gm.

Dinner: Meat, 60 gm.; 5 per cent vegetable, 135 gm.; Potato, 30 gm.; Cream, 40 cc.; Cheese, 15 gm.; Bread, 15 gm.; Butter, 25 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 135 gm.; Bacon, 10 gm.; Cream, 35 cc.; Cheese, 15 gm.; 10 per cent fruit, 50 gm.; Bread, 15 gm.; Butter, 27 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 50 cc. Forenoon—Olive oil, 30 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 16.

Breakfast: Eggs, 2; Bacon, 20 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 20 gm.; 10 per cent fruit, 80 gm.

Dinner: Meat, 60 gm.; Bread, 10 gm.; 5 per cent vegetable, 120 gm.; Potato, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.; Butter, 30 gm.; Watermelon, 45 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 120 gm.; Cheese, 15 gm.; Butter, 25 gm.; Bacon, 10 gm.; Cream, 60 cc.; Bread, 20 gm.; Watermelon, 45 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 20 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 17.

Breakfast: Eggs, 2; Bacon, 20 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 20 gm.; 10 per cent fruit, 80 gm.

Dinner: Meat, 60 gm.; Bread, 10 gm.; 5 per cent vegetable, 120 gm.; Potato, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.; Butter, 30 gm.; Watermelon, 45 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 120 gm.; Cheese, 15 gm.; Butter, 25 gm.; Bacon, 10 gm.; Cream, 60 cc.; Bread, 20 gm.; Watermelon, 45 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 20 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 18.

Breakfast: Eggs, 2; Bacon, 20 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 20 gm.; 10 per cent fruit, 80 gm.

Dinner: Meat, 60 gm.; Bread, 10 gm.; 5 per cent vegetable, 120 gm.; Potato, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.; Butter, 30 gm.; Watermelon, 45 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 120 gm.; Cheese, 15 gm.; Butter, 25 gm.; Bacon, 10 gm.; Cream, 60 cc.; Bread, 20 gm.; Watermelon, 45 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 20 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 19.

Breakfast: Eggs, 2; Bacon, 30 gm.; Bread, 10 gm.; Butter, 20 gm.; Cream, 30 cc.; Watermelon, 60 gm.

Dinner: Meat, 60 gm.; 5 per cent vegetable, 135 gm.; Cheese, 15 gm.; Butter, 34 gm.; Cream, 30 cc.; Watermelon, 60 gm.

Supper: Meat, 30 gm.; Bacon, 30 gm.; 5 per cent vegetable, 135 gm.; Cream, 30 cc.; Cheese, 15 gm.; Butter, 30 gm.; Watermelon, 60 gm.

Extras (one-third to each meal): Olive oil, 80 cc., Lemon, 15 cc.

Summary: Carbohydrate, 31.5 gm.; Protein, 63 gm.; Fat, 236 gm.

July 20.

Breakfast: Eggs, 2; Bacon, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Watermelon, 100 gm.

Dinner: Meat, 45 gm.; Cheese, 15 gm.; 5 per cent vegetable, 180 gm.; Butter, 30 gm.; Cream, 60 cc.; Watermelon, 65 gm.

Supper: Meat, 30 gm.; Bacon, 30 gm.; 5 per cent vegetable, 180 gm.; Cheese, 15 gm.; Butter, 27 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 31.5 gm.; Protein, 63 gm.; Fat, 236 gm.

July 21.

Breakfast: Eggs, 2; Bacon, 30 gm.; Butter, 25 gm.; Cream, 60 cc.; Watermelon, 100 gm.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 180 gm.; Cheese, 15 gm.; Butter, 30 gm.; Cream, 90 cc.; Watermelon, 65 gm.

Supper: Meat, 30 gm.; Bacon, 30 gm.; 5 per cent vegetable, 180 gm.; Cheese, 15 gm.; Butter, 27 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 31.5 gm.; Protein, 63 gm.; Fat, 236 gm.

July 22.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; Bread, 15 gm.; 10 per cent fruit, 40 gm.; Butter, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Cheese, 15 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Butter, 25 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 23.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Cheese, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; Cream, 60 cc.; 5 per cent vegetable, 90 gm.; Butter, 25 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Cheese, 15 gm.

Extras (one-third to each meal): Olive oil, 80 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 242 gm.

July 24.

Breakfast: Eggs, 2; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.; Bacon, 30 gm.; 10 per cent fruit, 100 gm.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Cheese, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; Cream, 60 cc.; Butter, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Cheese, 15 gm.

Extras (one-third to each meal): Olive oil, 40 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 202 gm.

July 25.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Cheese, 15 gm.; Bread, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Cheese, 15 gm.; Bread, 20 gm.; Butter, 25 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 26.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 25 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 35 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 27.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; 5 per cent vegetable, 90 gm.; Cream, 45 cc.; Bacon, 20 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Cream, 45 cc.; Bread, 35 gm.; Butter, 30 gm.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 28.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 29.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Cream, 45 cc.; Butter, 30 cc.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 30.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; Cheese, 15 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; Cream, 60 cc.; Cheese, 15 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Butter, 25 gm.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 31.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; Cheese, 15 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 30 gm.

Supper: Duck, 40 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; Cheese, 15 gm.; Bread, 20 gm.; Butter, 22 gm.; 10 per cent fruit, 55 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

Aug. 1.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; Cheese, 15 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Cheese, 15 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Butter, 25 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

Aug. 2.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; 5 per cent vegetable, 60 gm.; Bread, 30 gm.; Bacon, 25 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; 5 per cent vegetable, 60 gm.; Bread, 30 gm.; Potato, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 3.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 4.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; Potato, 30 gm.; 5 per cent vegetable, 60 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 5.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 30 cc.; Cream, 90 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 6.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 7.

Breakfast: Egg, 1; Egg white, 1; Bacon, 30 gm.; Oleo, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 200 gm.; Bread, 30 gm.; Milk, 60 cc.; Cream, 30 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 150 gm.; Potato, 30 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Cream, 20 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 150 gm.; Potato, 30 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Milk, 30 cc.; Cream, 30 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 8.

Breakfast: Egg, 1; Egg white, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 200 gm.; Oleo, 30 gm.; Bread, 30 gm.; Cream, 30 cc.; Milk, 60 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; Potatoes, 30 gm.; 5 per cent vegetable, 150 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Cream, 30 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 10 per cent fruit, 50 gm.; 5 per cent vegetable, 150 gm.; Oleo, 30 gm.; Potato, 30 gm.; Bread, 30 gm.; Milk, 30 cc.; Cream, 30 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 9.

Breakfast: Egg, 1; Egg white, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 200 gm.; Bread, 30 gm.; Oleo, 30 gm.; Cream, 30 cc.; Milk, 60 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; Potato, 30 gm.; 5 per cent vegetable, 150 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Milk, 60 cc.; Cream, 30 cc.

Supper: Egg, 1; Bacon, 30 gm.; 10 per cent fruit, 50 gm.; 5 per cent vegetable, 150 gm.; Oleo, 30 gm.; Bread, 30 gm.; Potato, 30 gm.; Milk, 30 cc.; Cream, 30 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 10.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Potato, 30 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Bread, 35 gm.; Butter, 25 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; Potato, 30 gm.; 5 per cent vegetable, 90 gm.; Bread, 35 gm.; Butter, 35 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

Aug. 11.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Potato, 30 gm.; Bacon, 20 gm.; 5 per cent vegetable, 150 gm.; Bread, 35 gm.; Butter, 25 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; Potato, 30 gm.; 5 per cent vegetable, 90 gm.; Bread, 35 gm.; Butter, 35 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

20 per cent cream was used. All food was weighed after cooking.

TABLE II

Case IV.

Date.	Diet.							Weight.	Alveolar CO_2.	Urine.						
	Protein.		Fat.		Carbohydrate.		Calories.			Volume.	Total N.	NH_4 N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*kg.*	*mm.*	*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
July 15	?	?	?	?	?	?	?	76.8		500	11.2	0.373	4.5	0.026	3.1	0.017
" 16	63	10	222	80	63	10	2,506	76.8	36	640	12.8	0.450	39.1	0.250	26.5	0.169
" 17	63	10	222	80	63	10	2,506	76.8	36	675	12.9	0.497	34.2	0.234	21.6	0.146
" 18	63	10	222	80	63	10	2,506	76.8		720	14.4	0.621	81.7	0.587	75.4	0.532
" 19	63	10	222	80	63	10	2,506		33	700	13.1	0.700	67.4	0.472	132	0.928
" 20	63	10	236	85	32	5	2,502		33	780	12.5	0.975	115	0.897	173	1.35
" 21	63	10	236	85	32	5	2,502	76.2	31	1,200	12.6	1.20	120	1.44	356	4.27
" 22	63	10	236	85	32	5	2,502	76.0	33	1,380	12.8	1.62	157	2.17	382	5.27
" 23	63	10	222	80	63	10	2,502	75.4	31	1,100	12.2	1.80	154	1.70	366	4.03
" 24	63	9.5	242	81	63	9.5	2,684		29	840	11.6	1.20	172	1.45	364	3.06
" 25	63	10.7	202	78.5	63	10.7	2,320			820		1.64	125	1.03	264	2 16
" 26	63	10	222	80	63	10	2,502	75.0	37	740	10.9	1.19	95.2	0.707	174	1.29
" 27	63	10	208	75	94	15	2,500		35	820	10.7	1.17	74.5	0.602	64.4	0.527
" 28	63	10	208	75	94	15	2,500	74.8	42	780	9.74	0.900	71.0	0.562	94.5	0.736
" 29	63	10	208	75	94	15	2,500		40	840	9.90	0.764	47.2	0.396	54.6	0.458
" 30	63	10	208	75	94	15	2,500		36	930	10.3	0.685	42.2	0.392	25.4	0.236
" 31	63	10	222	80	63	10	2,502	76.3	43	755	9.20	0.605	43.9	0.332	43.9	0.332
Aug. 1	63	10	222	80	63	10	2,502		40	780	9.77	0.600	117	0.917	70.3	0.542
" 2	63	10	222	80	63	10	2,502	76.0	41	830	10.4	0.602	78.0	0.608	110	0.914
" 3	63	10	194	70	125	20	2,498		40	925	10.1	0.514	64.1	0.594	60.8	0.561
" 4	63	10	194	70	125	20	2,498	76.3	44	1,060	9.48	0.623	46.0	0.495	78.2	0.827

Aug. 5	63	10	194	70	125	20	2,498	76.1		900	9.68	0.530	36.1	0.324	32.9	0.296
" 6	63	10	194	70	125	20	2,498	75.8	41	820			34.5	0.283	45.3	0.327
" 7	63	10	194	70	125	20	2,498	75.7	40	800	8.70	0.534	10.0	0.080	11.8	0.094
" 8	63	10	194	70	125	20	2,498		40	850	9.15	0.512	20.0	0.162	10.8	0.092
" 9	63	10	194	70	125	20	2,498	75.8	40	830	9.22	0.452	23.0	0.194	43.2	0.358
" 10	63	10	194	70	125	20	2,498		42	800	10.0	0.488	20.0	0.160	21.6	0.172
" 11	63	10	208	75	94	15	2,500			800	10.7	0.574	16.7	0.134	13.3	0.107
" 12	63	10	208	75	94	15	2,500	75.8		860	12.3	0.640	20.6	0.229	33.6	0.288

Results of determinations of the acetone bodies are expressed in terms of acetone. The subject upon whom this experiment was performed was the same as the one used for Experiment 1.

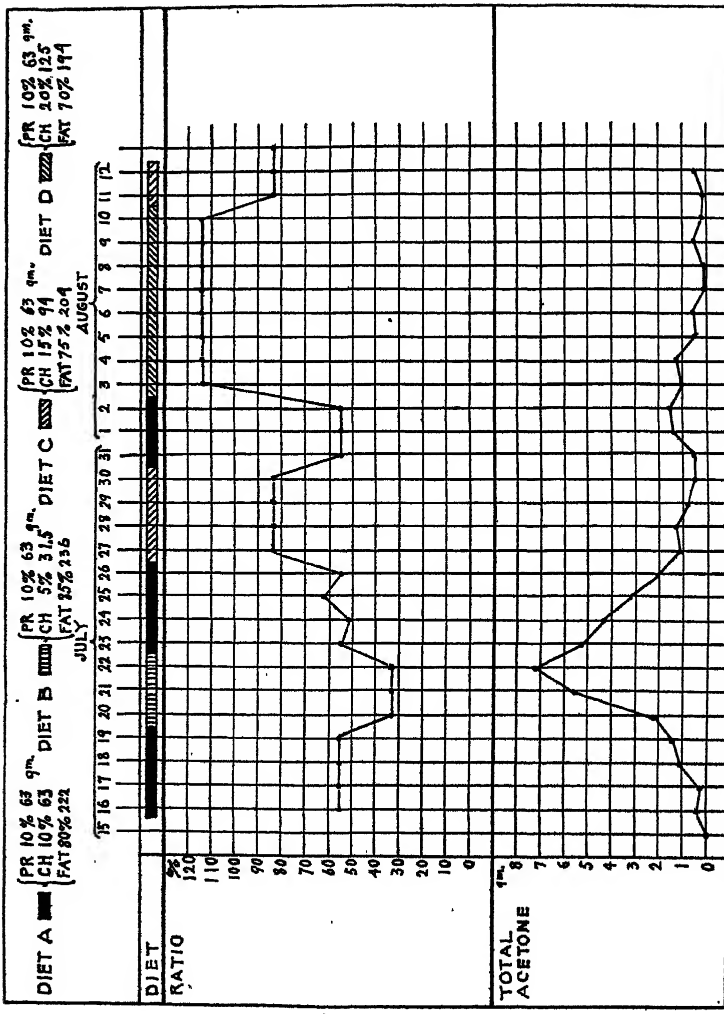

DIET A
PR 10% 63 gm.
CH 10% 63
FAT 80% 222
DIET B
PR 10% 63 gm.
CH 5% 31.5
FAT 85% 236
DIET C
PR 10% 63 gm.
CH 15% 94
FAT 75% 204
DIET D
PR 10% 63 gm.
CH 20% 125
FAT 70% 194
JULY
AUGUST
DIET
RATIO
TOTAL ACETONE

Chart 2.

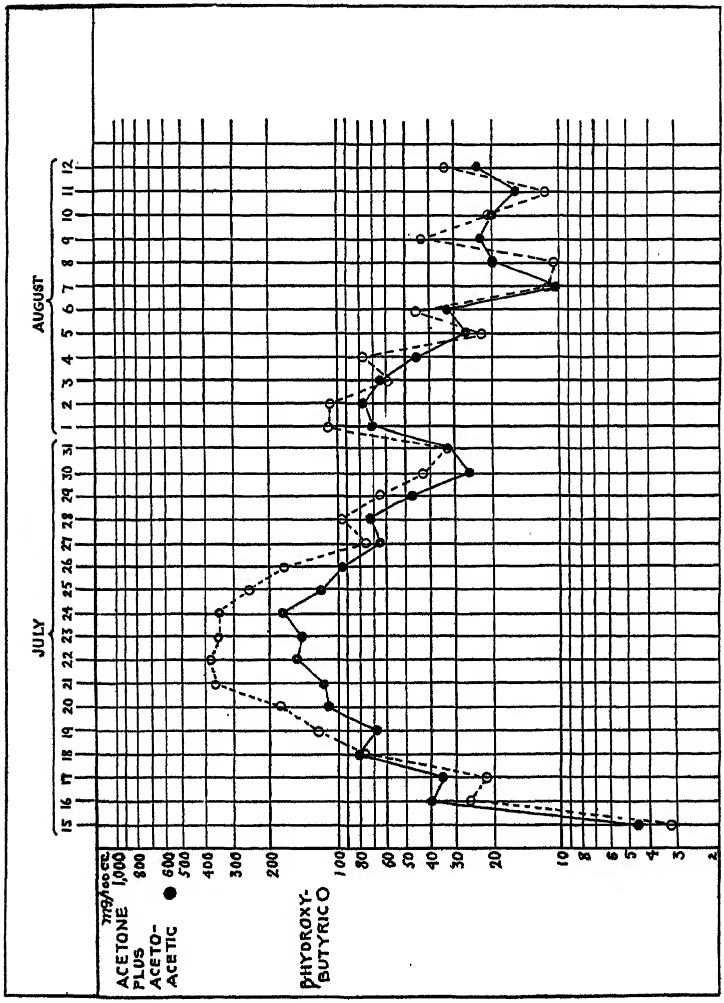

JULY
AUGUST
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 1 2 3 4 5 6 7 8 9 10 11 12
mg. per 100 cc.
1,000
800
600
500
400
300
200
100
80
60
50
40
30
20
10
8
6
5
4
3
2
ACETONE PLUS ACETO-ACETIC ●
β-HYDROXY-BUTYRIC ○

CHART 2A.

TABLE III

Case II

Date.	Diet.							Weight.	Urine.								
	Protein.		Fat.		Carbohydrate.		Calories.		Volume.	Reaction.	Acid.	Total N.	NH_4 N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*kg.*	*cc.*	*pH*	*cc. 0.1N*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
Feb. 16	?	?	?	?	?	?	?		1,570	6.0	358		0.295	0.2	0.007	0.5	0.009
" 17	36	9.5	133	81	36	9.5	1,478	68.5	1,730	5.2	299		0.250	0.8	0.013	0.7	0.013
" 18	36	9.5	133	81	36	9.5	1,478	68.0	1,760	5.75	226		0.226	3.7	0.067	2.5	0.044
" 19	36	9.5	133	81	36	9.5	1,478	67.7	2,140	5.3	361		0.306	6.6	0.142	5.1	0.110
" 20	36	9.5	133	81	36	9.5	1,478										
" 21	36	9.5	133	81	36	9.5	1,478	67.2	1,795	5.3	301		0.346	9.5	0.171	6.1	0.112
" 22	36	9.5	133	81	36	9.5	1,478	67.0	2,190	5.3	316	5.10	0.353	6.5	0.144	4.7	0.085
" 23	36	9.5	133	81	36	9.5	1,478	67.0	1,880	5.35	276		0.246	11.3	0.136	12.1	0.155
" 24	36	9.5	133	81	36	9.5	1,478	67.0	1,575	5.4	290		0.303	6.9	0.109	7.6	0.121
" 25	36	9.5	133	81	36	9.5	1,478	67.1	1,400	5.5	291	6.07	0.334	15.5	0.210	22.6	0.329
" 26	36	9.5	133	81	36	9.5	1,478	66.8	1,380	5.35	264	7.07	0.307	12.5	0.183	13.2	0.183
" 27	36	10	126	79.5	37	10.4	1,426	66.7									
" 28	36	10	126	79.5	37	10.4	1,426	66.4	1,240	5.4	253		0.285	7.8	0.104	8.6	0.096
Mar. 1	36	10	126	79.5	37	10.4	1,426	66.4	1,565	5.3	289	7.10	0.284	4.1	0.064	4.7	0.074
" 2	36	10	126	79.5	37	10.4	1,426	66.3	1,300	5.7	224	6.50	0.192	5.2	0.068	2.9	0.038
" 3	36	10	126	79.5	37	10.4	1,426	66.2	1,220	5.4	284		0.200	5.5	0.066	4.7	0.058
" 4	45	10	153	75.1	68	15	1,816	66.2	1,460	5.5	246		0.225	4.1	0.060	2.3	0 034
" 5	45	10	153	75.1	68	15	1,816										
" 6	18		90		59		1,122	66.0									
" 7	45	10	153	75.1	68	15	1,816	66.2									
" 8	45	10	153	75.1	68	15	1,816	66.2	1,395	5.3	280		0.269	1.7	0.024	2.0	0.028
" 9	45	10	153	75.1	68	15	1,816	66.2	1,300	5.45	260		0.260	2.2	0.028	2.3	0.030
" 10	45	10	153	75.1	68	15	1,816	66.2	1,385	5.45	276		0.281	1.0	0.014	1.0	0.013

Results of determinations on the acetone bodies are expressed in terms of acetone.

Case II was a woman, Miss A. G., aged 47 years, whose height was 5 ft. 2 in. and who weighed 137 lbs. Her basal metabolism measured 1,335 calories per day. Like all of the pathological cases included in the series, she was a severe chronic arthritic of long standing. She made every effort to cooperate, but after a little more than a week she found it difficult to take the basal diet, and it was accordingly modified as shown in Table III and Chart 3. The diet fed during the first part of the experiment did not contain enough food to maintain the weight of the patient, and there was a progressive loss during the first 2 weeks. In four out of five determina-

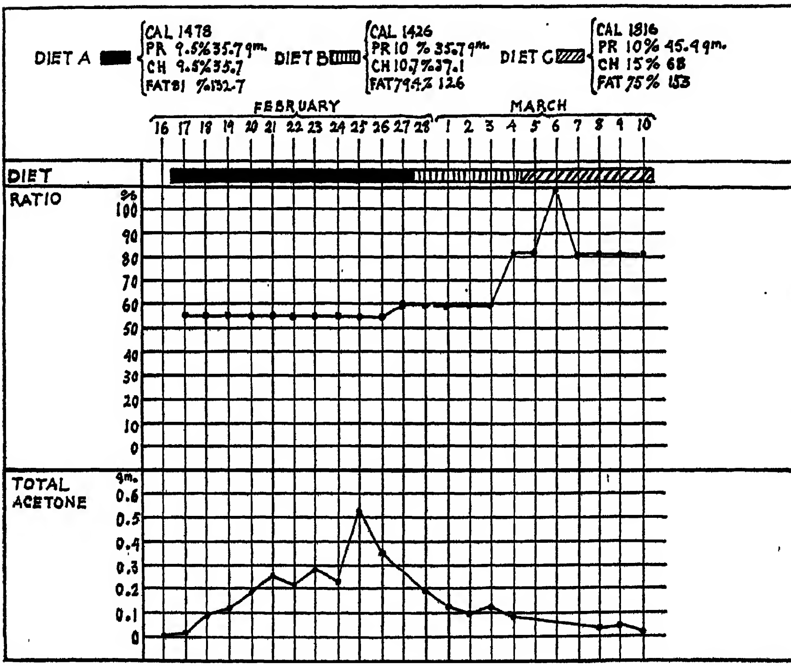


CHART 3.

tions of urinary nitrogen made during this period there was more found than was contained in the food taken, but on the day when the difference was largest, Mar. 1, the ratio used to express the ketogenic balance had a value of 56 per cent when calculated from the food fed and of 59 per cent when the protein burned was calculated from the urinary nitrogen; this difference is almost certainly within the limits of experimental error.

This case showed the smallest excretion of the acetone bodies of any in the series (the scale which has been used in plotting the acetone excretion is ten times as great as it is in the other cases), but still, when diets approximating the 10, 10, 80 per cent, diet were fed, the concentrations of

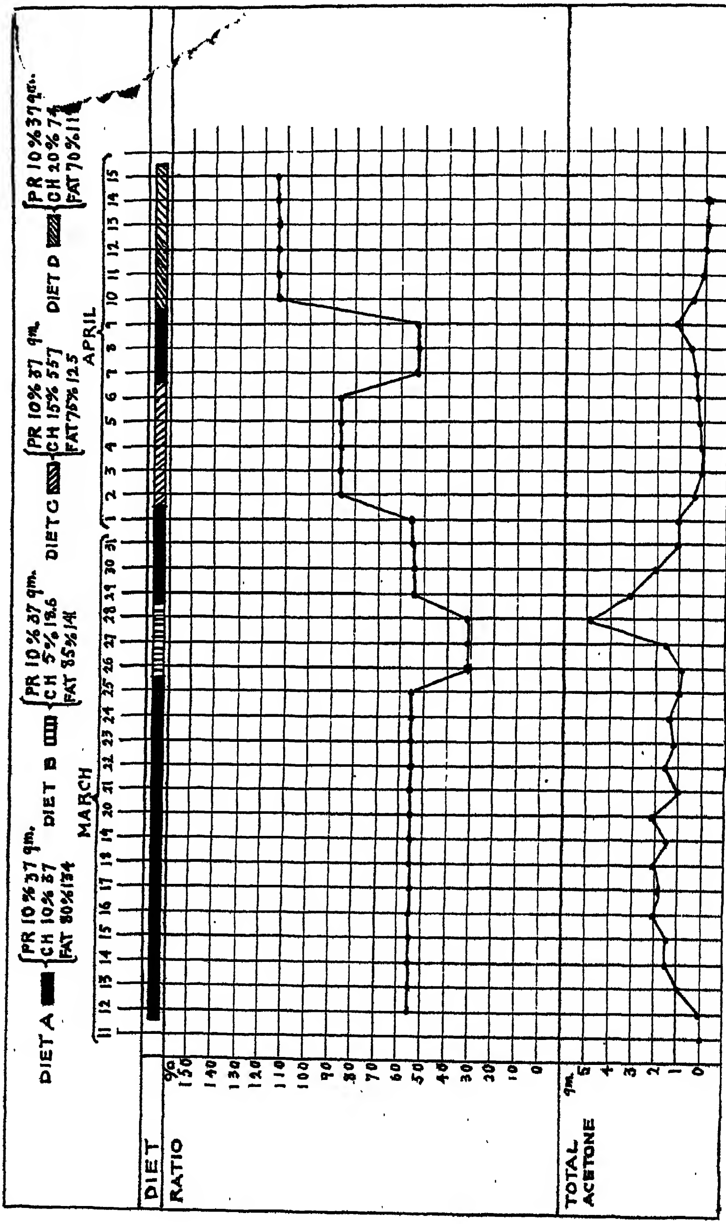
TABLE IV.

Case III.

Date.	Diet.								Weight.	Alveolar CO_2.	Urine.								
	Protein.		Fat.		Carbohydrate.		Calories.	$NaHCO_3$			Volume.	Reaction.	Acid.	Total N.	NH_4N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*gm.*	*kg.*	*mm.*	*cc.*	*pH*	*cc. 0.1N*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
Mar. 11	?	?	?	?	?	?	?		40.1		815	5.7	280	8.15	0.610	3.2	0.026	2.0	0.016
" 12	37	10	134	80	37	10	1,502		40.1	34	550	5.4	260	6.90	0.526	16.7	0.092	24 5	0.135
" 13	37	10	134	80	37	10	1,502			34	510	5.5	322	7.55	0.425	44.3	0.226	142	0.725
" 14	37	10	134	80	37	10	1,502		40.2	33	580	5.35	306	6.40	0.596	73.2	0.417	206	1.19
" 15	37	10	134	80	37	10	1,502		40.0	34	690	5.3	265	6.13	0.616	59.3	0.408	163	1.13
" 16	37	10	134	80	37	10	1,502	1.94	40.0	34	420	5.55	219	4.42	0.700	113	0.475	402	1.69
" 17	37	10	134	80	37	10	1,502	1.94	40.0	35	895	5.6	244	8.72	0.793	59.2	0.530	156	1.40
" 18	37	10	134	80	37	10	1,502	1.94	40.2	30	640	5.85	128	4.42	0.505	103	0.613	265	1.69
" 19	37	10	134	80	37	10	1,502	3.89	40.0	34	815	6.05	222	7.24	0.469	64.8	0.528	116	0.945
" 20	37	10	134	80	37	10	1,502	3.89		34	575	6.6	117	6.39	0.288	108	0.620	276	1.59
" 21?	37	10	134	80	37	10	1,502	3.89	40.2	37	340	6.3	130	4.45	0.219	90.7	0.308	209	0.713
" 22	37	10	134	80	37	10	1,502	3.89	40.4	37	675	6.3	199	7.20	0.374	88.2	0.595	165	1.12
" 23?	37	10	134	80	37	10	1,502		40.0	37	350	6.0	148	5.38	0 326	99.7	0.349	264	0.925
" 24	37	10	134	80	37	10	1,502		40.4	33	800	5.5	282	6.66	0.547	57.4	0.459	114	0.914
" 25	37	10	134	80	37	10	1,502		40.0		730	5.7	198	5.42	0.456	46.3	0.338	95.6	0.698
" 26?	37	10	141	85	19	5	1,493		40.2	30	320	5.7	153	3.66	0.426	88.9	0.284	209	0.670
" 27?	37	10	141	85	19	5	1,493			27	380	5.7	176	5.42	0.574	209	0.458	290	1.10
" 28	37	10	141	85	19	5	1,493		40.0	31	685	5.7	307	9.78	0.976	168	1.15	568	3.88

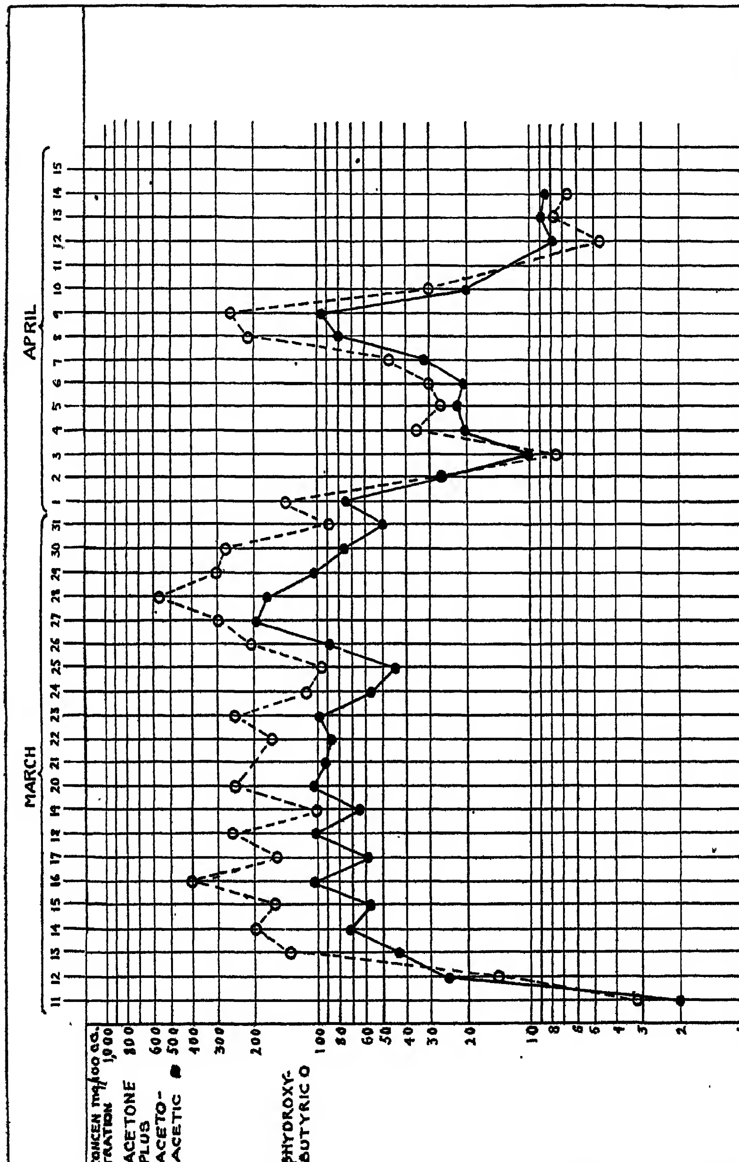
Mar. 29	37	10	134	80	37	10	1,502			28	820	5.6	296	7.46	1.32	103	0.851	315	2.58
" 30	37	10	134	80	37	10	1,502			30	840	5.55	255	7.48	1.10	74.3	0.625	187	1.57
" 31	37	10	134	80	37	10	1,502			31	760	5.7	231	5.44	0.760	49.2	0.374	87.5	0.666
Apr. 1	37	10	134	80	37	10	1,502				510	5.9	192	6.81	0.574	74.2	0.378	143	0.798
" 2	37	10	125	75	56	15	1,495				960	5.9	231	6.10	0.514	25.9	0.248	26.4	0.254
" 3	37	10	125	75	56	15	1,495			32	840	6.2	168	6.70	0.457	10.0	0.084	6.9	0.058
" 4	37	10	125	75	56	15	1,495			31	325	5.85	197	4.62	0.358	21.0	0.068	35.5	0.115
" 5	37	10	125	75	56	15	1,495			29	555	5.84	244	5.84	0.347	21.8	0.121	26.6	0.147
" 6	37	10	125	75	56	15	1,495			33	595	5.8	238	5.67	0.356	20.7	0.123	30.0	0.178
" 7	37	9.5	141	81	37	9.5	1,565			33	570	5.85	246	5.32	0.518	31.5	0.178	46.7	0.266
" 8?	37	9.5	141	81	37	9.5	1,565			33	320	5.7	179	4.00	0.320	80.4	0.257	217	0.695
" 9	37	9.5	141	81	37	9.5	1,565			35	620	5.55	303	6.20	0.620	93.2	0.573	256	1.59
" 10	37	10	117	70	75	20	1,501			36	860	5.55	324	5.50	0.384	22.5	0.190	30.7	0.263
" 11	37	10	117	70	75	20	1,501			35	825	5.9	250	5.33	0.292	16.2	0.133	17.7	0.145
" 12	37	10	117	70	75	20	1,501			36	740	6.25	207	5.09	0.284	6.7	0.050	4.4	0.033
" 13	37	10	117	70	75	20	1,501			36	715	6.1	211	4.98	0.286	9.0	0.063	7.7	0.050
" 14	37	10	117	70	75	20	1,501			36	520	6.2	192	4.42	0.260	8.6	0.045	6.4	0.033
" 15	37	10	117	70	75	20	1,501		40.9										

Results of determinations of the acetone bodies are expressed in terms of acetone.



DIET A
PR 10% 37 gm.
CH 10% 37
FAT 80% 134
DIET B
PR 10% 37 gm.
CH 5% 18.6
FAT 85% 141
DIET C
PR 10% 37 gm.
CH 15% 55.7
FAT 75% 125
DIET D
MARCH
APRIL
DIET
RATIO
TOTAL ACETONE

CHART



MARCH
APRIL
CONCEN TRATION mg/100 cc.
ACETONE PLUS ACETO-ACETIC ●
β HYDROXY-BUTYRIC O

CHART 4A.

the acetone bodies varied from ten to one hundred times the values of those found when the subject was on a normal diet. It seems possible that the continued loss of weight and negative nitrogen balance noted in this case may be accompanied by a liberation of glycogen which would serve as an additional (endogenous) source of antiketogenic compounds, but as other experiments, as Case IV, do not show evidences of such a process during loss of weight this explanation can only be offered tentatively. The figures actually obtained show that the border-line diet was very close to the basal one, and as this is the only case in the series in which this was found to be true, it seems reasonable that some such phenomena as those suggested above may have diminished the excretion. The results show also that the ratio between the fat and carbohydrate rather than the amount of fat ingested determines the acetone excretion, for less acetone was found when the diet contained 153 gm. of fat and 45 gm. of carbohydrate than when it contained 133 gm. of fat and 36 gm. of carbohydrate. The excretion of the acetone bodies was so low that the determination of creatinine was not interfered with, and the determinations of this compound showed a high degree of success in collecting accurate 24 hour urines.

Case III, Mrs. M. H., was a woman 28 years old, 5 ft. 3 in. tall, and weighing 70 lbs. Her basal metabolism measured 1,240 calories per day. She had been suffering from severe chronic arthritis for 2 years, and was practically helpless. A special nurse was assigned to the case, and both the nurse and the patient cooperated well in carrying out directions. Altogether the results of the study of this case were very satisfactory, but in some instances specimens of urine were unavoidably lost due to the condition of the patient; the days on which these losses occurred are marked in the table with an interrogation point. The patient ate the entire amount of the diet provided at all times, and the diet furnished maintained the body weight throughout the experiment. It was possible to continue the base line diet long enough to determine the excretion of acetone caused by it with more accuracy in this case than in any other. Diets which had a lower ratio of carbohydrate to fat than did the basal diet caused a formation of larger amounts of acetone, and the change from one level of excretion to the other was gradual and not abrupt.

A diet which had a ketogenic power of 108 per cent caused practically no increase in the excretion of acetone; one having a value of 78 per cent caused an excretion of distinctly increased amounts, although these amounts were not great; while the basal diet—which has a value of 55 per cent—caused an excretion of between 1 and 2 gm. From these figures it would seem that the value of the border-line diet must lie at about 78 per cent, unless considerable importance is attached to the formation of very slight traces of the acetone bodies.

In this case small amounts of sodium bicarbonate were fed over a period of a few days after acetone excretion was thought to have reached an equilibrium which corresponded to the basal (10, 10, 80 per cent) diet. The sodium bicarbonate lowered the excretion of ammonia and of titra-

TABLE V.

Case V.

Date.	Diet.							Weight.	Alveolar CO_2.	Urine.						
	Protein.		Fat.		Carbohydrate.		Calories.			Volume.	Total N.	NH_4N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*kg.*	*mm.*	*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
July 30	?	?	?	?	?	?	?			690		0.314	0.8	0.005	2.9	0.020
" 31	45	8.9	184	82.2	45	8.9	2,016	57.3	43	770	5.05	0.306	9.2	0.071	12.6	0.098
Aug. 1	46	9.1	181	81.8	45	9.1	1,989			820	8.10	0.586	48.2	0.394	89.7	0.734
" 2	45	9.5	170	81	45	9.5	1,890	57.0		830	7.20	0.518	83.0	0.688	153	1.27
" 3	45	10.5	149	79	45	10.5	1,701			800	5.70	0.488	92.0	0.735	178	1.42
" 4	45	9.6	167	80.8	45	9.6	1,879	56.2		770	6.70	0.736	120	0.924	307	2.46
" 5	45	8.9	174	77.3	72	13.8	2,036		36	500	5.00	0.500	129	0.645	458	2.29
" 6	45	8.8	176	77.5	72	13.7	2,052	56.4		620			122	0.754	371	2.30
" 7	45	9.0	170	77	72	14	1,998		36	440	3.86	0.478	80.6	0.360	410	1.80
" 8	45	8.7	178	77.8	72	13.5	2,070	56.5		540	5.00	0.660	86.6	0.467	188	0.865
" 9	45	8.9	153	69.1	117	23	2,025			570	3.00	0.324	40.0	0.188	51.8	0.296
" 10	45	8.0	178	71.2	117	20.8	2,250		38	180	1.50	0.150	44.8	0.081	41.1	0.074
" 11	45	8.0	178	71.2	117	20.8	2,250	57.4		700	2.90	0.188	55.0	0.035	7.8	0.055
" 12	45	10	150	64	67	26	1,800	56.8		260	1.49	0.100	38.3	0.100	29.4	0.076
" 13	35	9.6	121	59.1	67	31.3	1,497		34							
" 14	45	10.3	149	61.9	67	27.8	1,746									
" 15	45	10.3	150	63	67	26	1,800	57.0		500			46.7	0.234	38.3	0.191
" 16	47	10.2	152	64.2	69	25.6	1,832		34	390		0.108	25.0	0.098	28.4	0.111
" 17	46	10.2	149	63.7	68	26.1	1,797	57.3		570	4.06	0.365	25.8	0.147	31.4	0.179
" 18	45	10	150	63.9	68	26.1	1,800	57.2		340	2.72	0.243	81.5	0.277	165	0.563
" 19	45	10	150	63.9	68	26.1	1,800		41	410		0.293	60.0	0.246	109	0.563
" 20	46	10.2	138	62.2	67	27.6	1,699			530			49.2	0.261	89.4	0.411
" 21	?	?	?	?	?	?	?	57.6								

Results of determinations on the acetone bodies are expressed in terms of acetone.

table acid, diminished the degree of acidity of the urine, and increased the tension of carbon dioxide in the alveolar air. The amount of the acetone bodies and their concentration in the urine were increased while the subject received the drug, and returned to the level previously established after it was discontinued. The periods before and after the alkali was given were short, but the changes were so marked that the experiment probably indicates a real increase in the excretion of these compounds. The results are similar to those recorded by Joslin[4] and by Forssner (1911). As this was the only case in the series in which the effect of the administration of

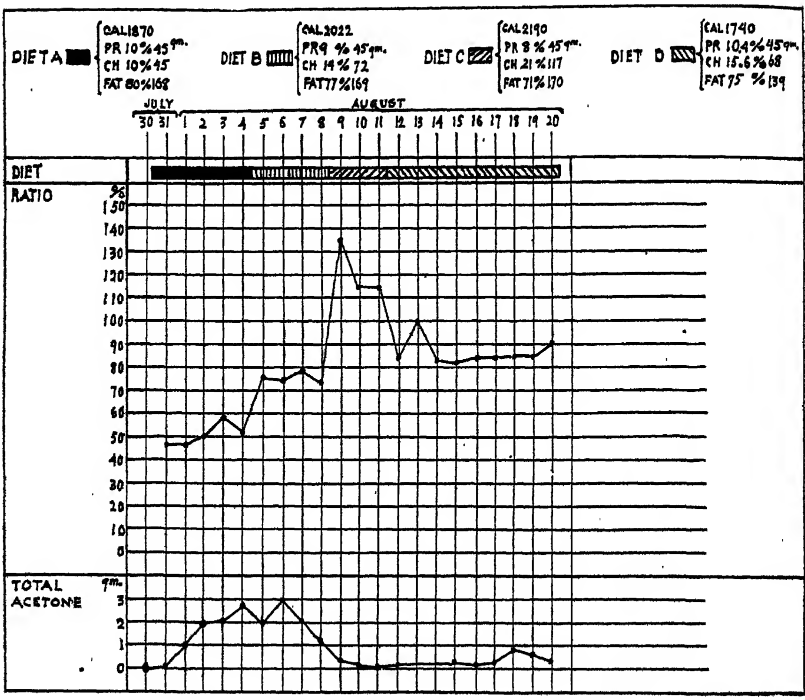


CHART 5.

alkali was studied, it is impossible to do more than note what may be an accidental finding.

Case V, Miss M. G., was a woman 22 years old, 5 ft. 2½ in. in height, who weighed 127 lbs. Her basal metabolism measured 1,430 calories per day. This experiment was much less satisfactory than those described above; the patient failed to eat all of the food provided in any of the diets, and also, apparently, to collect 24 hour urines in a satisfactory manner.

[4] Joslin (1917), pp. 394 and 395.

TABLE VI.

Case VI.

Date.	Diet.							Weight.	Urine.						
	Protein.		Fat.		Carbohydrate.		Calories.		Volume.	Total N.	NH_4 N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*kg.*	*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
July 12	50	16	90	64.8	60	19.2	1,250		1,240			42.0	0.522	11.6	0.144
" 13	50	16	90	64.8	60	19.2	1,250	57.4	890		0.371	22.2	0.197	70.7	0.630
" 14	45	14.7	90	66.4	58	18.9	1,222								
" 15	45	14.7	90	66.4	58	18.9	1,222		870			23.3	0.203	19.7	0.171
" 16	45	14.7	90	66.4	58	18.9	1,222		1,055					19.7	0.207
" 17	46	15.6	86	65.7	55	18.7	1,178		1,000			25.0	0.250	25.5	0.255
" 18	47	16.1	84	64.7	56	19.2	1,168	57.5							
" 19	35	13.4	76	65.5	55	21.1	1,044		1,200	10.5	0.350	15.8	0.190	13.7	0.165
" 20	49	16.8	82	63.1	59	20.1	1,170		1,220			17.5	0.214	23.6	0.288
" 21	50	16	90	64.8	60	19.2	1,250		1,590			18.3	0.292	25.4	0.404
" 22	47	14.2	86	65.6	60	20.2	1,182		1,545			21.7	0.336	42.2	0.654
" 23	50	16	90	64.8	60	19.2	1,250		1,000			15.8	0.158	24.6	0.246
" 24	50	16	90	64.8	60	19.2	1,250		1,800			20.8	0.374	32.3	0.583
" 25	50	16.1	90	65.3	58	18.6	1,242		1,060			56.3	0.602	23.4	0.248
" 26	40	12.8	90	64.8	70	22.4	1,250	57.3	720			52.2	0.376	47.7	0.344
" 27	40	12.8	90	64.8	70	22.4	1,250		820			39.8	0.326	53.7	0.440
" 28	41	13.1	90	64.6	70	22.3	1,254		680			41.6	0.282	15.6	0.106
" 29	40	12.9	90	65	69	22.1	1,246		1,240		0.516	13.3	0.165	11.7	0.145
" 30	40	12.8	90	64.8	70	22.4	1,250		1,360			14.1	0.192	26.5	0.358

Results of determinations of the acetone bodies are expressed in terms of acetone.

Although the weight varied somewhat from day to day it was fairly well sustained throughout the period of study. It seems probable in such a case that fat not taken in the diet is replaced in metabolism by tissue fat, although the possibility of an increased combustion of glycogen must also be considered. This case showed acetonuria when the diet had a ketogenic balance of about 80 per cent, and the acetonuria practically cleared up when a diet having a balance of 110 per cent was fed.

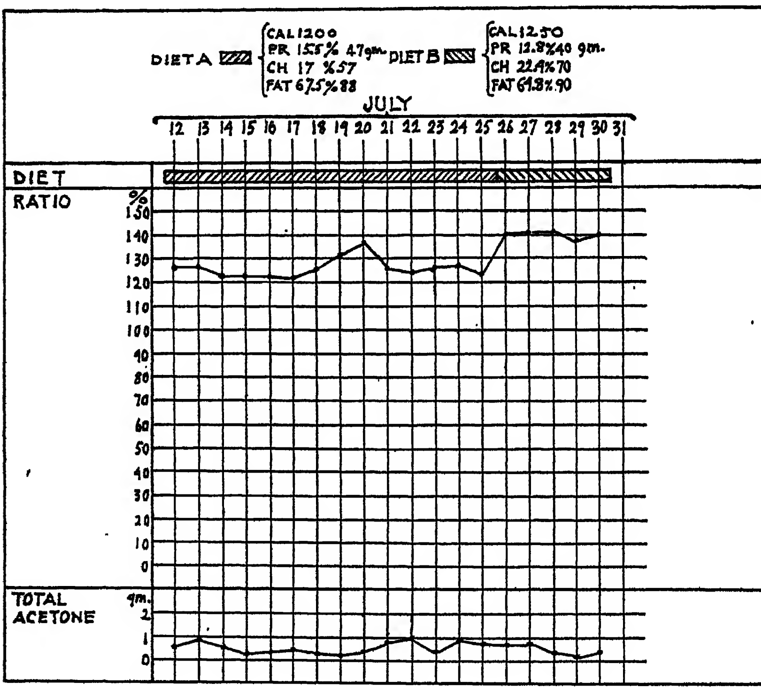


CHART 6.

Case VI, Mrs. E. Y., was a woman 70 years old, 5 ft. 2½ in. tall, who weighed 135 lbs. Her basal metabolism measured 1,185 calories per day. There was a special nurse assigned to the case, but there was a lack of cooperation because the patient had a prejudice against protein food, and disliked large quantities of fat. This case was even less satisfactory than the preceding one. When the series of experiments was commenced the patient had been living on a diet low in carbohydrate for some time as a part of treatment for chronic arthritis. Acetone was found in her urine by the qualitative test used (Legal's) when the diets did not seem to be severe enough to cause the presence of the compound, and it seemed desira-

ble to determine whether there was an increased elimination of both the acetone bodies. There is no doubt that the patient showed such an increase, although no strictly normal values are available for comparison. The diet taken during the period of study contained, on the average 15 per cent of the calories as protein, 17 per cent as carbohydrate, and 68 per cent as fat, and had a ketogenic balance of 120 to 130 per cent. The results are different from those found on other subjects, and may perhaps be attributed to changes in the metabolism of the patient caused by her advanced age.

There is one fact which is evident in all of the experiments; when the diet was changed the level of the acetone excretion changed to correspond, but changed gradually. Why these changes should have taken 3 or 4 days in some instances cannot be explained in an entirely satisfactory way. One factor which delayed the response was undoubtedly the time which it took ingested fat to pass through the digestive, assimilative, and metabolic processes, but this did not seem adequate to account for the delay completely. It is possible that when there was a large excess of ketogenic compounds included in the diet, glycogen or other antiketogenic materials were furnished from the reserve supplies of the body in larger amounts than normal. If this was so not only would the changes be gradual, as was found to be the case, but also the amounts of acetone found during the period for which a given diet was fed would be lower than that expected from a calculation of the ketogenic balance of the diet. The data reported above are not sufficient to decide this question. Whatever may have been the cause of this gradual change in the acetone excretion, there are three facts which result from it: first, no decision concerning the acetone excretion which corresponds with a diet can be made until the diet has been fed for several days; second, analyses of the fat content of stools is not necessary because it would not be possible to decide to what acetone excretion the figures would apply; third, it is useless to feed the diets—at least to feed the fat content of the diets—in small amounts taken frequently.

In interpreting the meaning of the excretion of acetone when diets are fed, which are at or near the border-line of ketogenic antiketogenic equilibrium, there are certain possibilities which must be kept in mind. For instance, the body tissues may furnish part of the material burned, and this will be of ketogenic or

as fat was the food which was left untouched, carbohydrate was the food which the subjects most craved, and a negative nitrogen balance was observed more frequently than was a positive one; the effect of the loss in weight and of the failure to collect urines accurately cannot be determined. A detailed examination of these sources of error has shown that either they could not be wholly avoided, or that their influence on the results was slight.

The difficulties which affect the interpretation of the excretion of the acetone bodies influence the study of the results of the experiments more than do the other uncertainties met with in these experiments. These sources of uncertainty include: temporary production of the acetone bodies due to variations in the foodstuffs burned at different times during the day; local production of the acetone bodies caused by variations in different parts of the organism; and, possibly, the effect of glycogen drawn from the reserve stores of the body. All of these except the last would lead to a production of acetone greater than the composition of the diets would indicate.

In interpreting the value of the expression

$$100 \times \frac{1.5\ (\text{weight carbohydrate} + 25 \text{ per cent weight protein})}{95 \text{ per cent weight fat}} = \text{N per cent}$$

which will express the condition of ketogenic antiketogenic equilibrium, the effect of these uncertainties, particularly of those affecting the interpretation of small amounts of the acetone bodies, must be kept in mind. When the value of the expression was 100 per cent or more, acetone was not found in the urine except in very small amounts, and in two of the cases studied, the excretion decreased when the diets had this value to the normal level. In one other case such a diet failed to cause the appearance of a distinctly increased acetonuria, although the period of study (3 days) was perhaps not long enough to produce an equilibrium in the body. When diets were fed which gave numerical values between 55 and 60 per cent rather large amounts of acetone were excreted; there was a distinctly increased excretion also, except in Case II, when diets giving values of about 80 per cent were taken. It seems most reasonable to attribute the small amounts of acetone found on the diets which figured at 100 per cent to local and temporary production of the acetone bodies, and to conclude that values of

80 to 90 per cent approximately represent the diet in which the ketogenic and antiketogenic foods are present in equivalent amounts.

It has been shown already that certain numerical values of the expression when ketogenic equilibrium is attained correspond to the different possible antiketogenic effects of the glycerol radicle: if glycerol does not figure as a source of antiketogenic compounds the value is 100 per cent; if glycerol is converted into glucose, and this glucose takes part in the reaction between ketogenic and antiketogenic compounds, the value is 83 per cent; if glycerol takes part in the reaction as a three carbon atom residue, the value is 67 per cent. A comparison of these values with the one which has been found experimentally to correspond with the condition of equilibrium makes it seem most probable that the glycerol residue of the fats does figure only to the extent to which it can yield glucose. These conclusions would be expressed mathematically as follows:

$$100 \times \frac{1.5 \text{ (weight carbohydrate + 25 per cent weight protein)}}{\text{95 per cent weight fat}} = \text{83 per cent}$$

If this equation is transposed so as to express the amounts of protein, fat, and carbohydrate which should be fed to produce a condition of ketogenic equilibrium, the expression becomes:

$$1.9 \text{ (weight carbohydrate + 25 per cent weight protein) = fat.}$$

This expression is practically identical with that stated by Woodyatt (1921):[5] "2 × carbohydrate + protein = fat."

It is of course possible that too much stress has been laid upon "temporary" and "local" sources of traces of acetone, and that not enough emphasis has been placed upon glycogen as a source of antiketogenic compounds. If very small amounts of acetone result from an excess of antiketogenic material in the diet, 100 per cent probably represents the condition of equilibrium. In this case the expression would be:

$$100 \times \frac{1.5 \text{ (weight carbohydrate + 25 per cent weight protein)}}{\text{95 per cent weight fat}} = \text{100 per cent}$$

and the expression for the relative amounts of food would become:

[5] Woodyatt (1921), p. 133.

1.42 (weight carbohydrate+25 per cent weight protein) = weight fat.

This expression probably does not express the condition of ketogenic equilibrium correctly; the one given above is almost certainly preferable.

It seems reasonable to conclude from the experiments reported that the two and three carbon atom residues from the α-amino-acids do not figure directly in the antiketogenic reaction, but are condensed to glucose. If these residues did react with the ketogenic compounds the numerical value for each diet would be higher than they are reckoned here; acetonuria would develop and clear up at values of from 100 to 120 per cent, and traces of acetone would be found in some cases when the value was 150 per cent.

The charts and tables recording these experiments have been examined for evidence of an adaptation of the organism to these diets high in fat with a consequent reduction of the amounts of the acetone bodies excreted. Folin and Denis (1915) have reported evidences of such an adaptation to starvation in three obese women studied by them, but there did not seem to be such a response to diets high in fat. When the basal diet—containing 10 per cent of the calories in the form of protein, 10 per cent in the form of carbohydrate, and the balance in the form of fat—was resumed after periods during which diets containing relatively more or less fat was fed, the excretion of acetone returned to the level first established if the base line diet was continued over a sufficient period.

The method adopted of plotting the concentration of the acetone bodies upon paper ruled with logarithmic characteristics shows clearly the relationship between the two fractions of the acetone bodies discussed in an earlier paper (Hubbard, 1921). When large amounts of the acetone bodies were excreted the acetone from β-hydroxybutyric acid was in excess of that from preformed acetone plus acetoacetic acid, but when the concentrations were only slightly increased the two fractions were as a rule nearly equal; in some cases the acetone from preformed acetone plus acetoacetic acid was in excess. When acetonuria developed slowly it was this fraction which increased first, while the β-hydroxybutyric acid increased later. The interpretation of these facts is complicated by differences in the kidney thresholds of the different acetone bodies.

Other results which have not yet been discussed include changes in alveolar carbon dioxide tension, the excretion of ammonia and of titratable acid, and changes in the reaction of the urine. The urinary ammonia roughly paralleled the acetone bodies except when sodium bicarbonate was added to the diet; during that period the excretion of ammonia was markedly reduced, while that of the acetone bodies was somewhat increased. The alveolar carbon dioxide tension was somewhat lowered by the more extreme diets, and the values returned to normal when sodium bicarbonate was taken. The variations of the titratable acidity and hydrogen ion concentration were little greater than those which are normally found; these were, of course, markedly affected by the administration of the alkali.

CONCLUSION.

A method has been suggested for expressing the ketogenic balance of any diet mathematically. A series of six experiments has been described in which the effect of diets high in fat on the excretion of the acetone bodies by normal subjects was studied, and the results compared with this mathematical expression. From the results obtained the following conclusions have been drawn: (1) that the mechanism which controls the formation of increased amounts of the acetone bodies can be regarded as a molecular reaction or balance between ketogenic substances such as the fatty acids and antiketogenic substances such as glucose; (2) that protein figures as an antiketogenic compound only to the extent of the glucose which it can yield in the organism; (3) that glycerol, when fed as a part of the fat molecule figures as an antiketogenic compound only to the extent to which it forms glucose in the organism; and (4) probably that glycerol so fed does figure as an antiketogenic compound to the extent to which glycerol itself can yield glucose.

Our thanks are due to Dr. Philip A. Shaffer for suggestions offered and for encouragement extended during the progress of the work described.

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THE RESOLUTION OF HYDROXYASPARTIC ACIDS INTO OPTICALLY ACTIVE FORMS.

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In a recent communication the synthesis and separation of two inactive forms of hydroxyaspartic acid have been described (1). The inactive form, more soluble in water, gave mesotartaric acid on treatment with nitrous acid and was designated as the *anti* compound, while the less soluble form gave racemic acid under similar conditions and was named the *para* compound. Each of these inactive acids contains two dissimilar asymmetric carbon atoms and should be resolvable into active components, giving a total of four active and two inactive forms. The resolution of the *anti* acid was readily effected by means of alkaloids as described in the present communication, but the *para* acid could not be resolved by this method although its finely crystalline alkaloid salts were subjected to exhaustive fractional crystallization. It appears that the alkaloid salts of the *para* acid are partially racemic compounds of the type described by Ladenburg (2). On turning to alternative biological methods for the resolution of the *para* acid it was found that no resolution could be effected by growing *Penicillium glaucum* in solutions of the sodium salt while some rather inconclusive evidence was secured of a slight resolution by fermenting yeast used according to Ehrlich's method (3). The small amount of dextro-rotatory acid thus obtained gave dextro-tartaric acid on treatment with nitrous acid. Since the Walden inversion rarely occurs with nitrous acid it is probable that *d*-hydroxyaspartic acid and *d*-tartaric acids are similarly constituted, and the same would be true of the *levo* forms. Both active forms of *anti*-hydroxyaspartic acid give inactive mesotartaric acid on treatment with nitrous acid so that their relative configuration remains undecided. On heating either of the active *anti* acids with water at 125° partial conversion into the

para acid was effected, but the latter was invariably optically inactive.

It is perhaps somewhat surprising that the active *anti*-hydroxyaspartic acids should have as high a specific rotation as 12°. In the light of various theories of optical superposition it might be anticipated that a substance derived from internally compensated mesotartaric acid by the replacement of one hydroxyl group with a relative mass of 17 by an almost equally heavy amino group with a mass of 16, would have a vanishingly small rotation but this is evidently not the case. Of course the possibility still exists of a Walden inversion taking place in the action of nitrous acid on hydroxyaspartic acid, but at present the evidence is against such an assumption.

Resolution of Inactive Anti-Hydroxyaspartic Acid.

The inactive *anti* acid, prepared as previously described, gives well crystallized salts with quinine, brucine, and strychnine and resolution may be effected by fractional crystallization of any of them. On the whole the most satisfactory plan is to separate the dextro acid first as strychnine salt and to use quinine for the separation of the levo acid. The morphine, cinchonine, and quinidine salts were not found helpful for purposes of resolution.

Strychnine d-Anti-Hydroxyaspartate.—10 gm. of *anti*-hydroxyaspartic acid were heated on a water bath with 75 cc. of water and slightly less than the theoretical amount of strychnine was added by degrees. The clear solution was then set aside to crystallize in an ice box. The salt crystallized readily in laminated plates which were filtered off and washed with 50 per cent aqueous acetone. The yield of crude strychnine salt is approximately the theoretical amount calculated for the dextro component and was obtained apparently optically pure after two further crystallizations from water (50 cc.). The air-dried salt contains close to 4 molecules of water which were removed on drying at 120° over phosphorus pentoxide under greatly reduced pressure. The salt is insoluble in acetone and moderately soluble in methyl and ethyl alcohol. It crystallizes best from water in which it is very soluble when hot, but sparingly soluble at low temperature.[1]

[1] The optical rotations recorded in this paper were observed in a highly sensitive Schmidt and Haensch polarimeter, for the use of which instrument I am indebted to The Rockefeller Institute for Medical Research.

0.1430 gm. air-dried salt lost 0.0183 gm. H_2O at 120° = 12.9 per cent.
Calculated for $C_{21}H_{22}N_2O_2 \cdot C_4H_7O_5N \cdot 4H_2O$ = 13.0 " "
Rotation. c = 1.0 air-dried salt in water; l = 2.2; $\alpha = -0.42°$
$[\alpha]_D^{20°} = -19.1°$

d-Anti-Hydroxyaspartic Acid.—The strychnine salt obtained as just described was dissolved in hot water and the bulk of the strychnine precipitated with a slight excess of ammonia. The filtrate was then extracted repeatedly with a mixture of amyl alcohol and ether until the remaining trace of strychnine was removed. The solution was then concentrated under diminished pressure to a small bulk (15 cc.) and rendered just acid to Congo red by the cautious addition of dilute nitric acid. On allowing the solution to stand in a cool place the dextro acid separates out readily in the form of transparent thick wedge-shaped prisms which only become opaque after long standing. For analysis the acid was dried over phosphorus pentoxide at 60°.

0.1656 gm. substance : 0.1971 gm. CO_2, 0.0724 gm. H_2O.
$C_4H_7O_5N$. Calculated. C 32.2, H 4.7.
Found. " 32.5, " 4.8.
Rotation. c = 2.0 in water; l = 2.2; $\alpha = +0.53°$
$[\alpha]_D^{20°} = +12.1°$

The dextro acid is slightly less soluble in water than the inactive acid, dissolving in about 45 parts of water at room temperature compared with about 30 parts for the latter. The chemical properties of the active acid as expected closely resemble those of the inactive acid. An interesting fact was noted that on recrystallizing a mixture of the dextro and inactive acids from water, the mother liquor which was at first dextro-rotatory, on standing in contact with the separated crystals gradually became entirely inactive while the separated dextro acid increased in amount. It is inferred that the inactive acid is a *dl*-mixture at any rate in solution at room temperature and not truly racemic, since under these circumstances the shifting of the equilibrium is readily comprehensible. The yield of pure dextro acid from 10 gm. of the inactive compound was 3.2 gm. A slightly larger yield may be obtained by separating the acid as lead salt rather than by direct crystallization, but the optical purity of the product is apt to be impaired. The optical rotation of the dextro acid is increased about 30 per cent on addition of hydrochloric acid. Most of the soluble salts are also dextro-rotatory.

Quinine l-Anti-Hydroxyaspartate.—The mother liquor from the strychnine salt of the dextro acid may be utilized conveniently for the preparation of this salt although it may also be obtained direct from the inactive acid. The strychnine mother liquors are precipitated with ammonia, and a crude levo acid obtained by neutralizing the concentrated filtrate with nitric acid using Congo red as indicator. The acid crystallizes readily on keeping in a cool place.

The crude levo acid (6 gm.) was suspended in 75 cc. of water and quinine base (15 gm.) added by degrees while heating on the water bath. The clear solution on cooling quickly deposited long needles of the salt which were filtered off on the following day. The yield of air-dried salt was 12.7 gm. and its specific rotation was −96.4. On recrystallization from water (20 cc.) the rotation was practically constant at −95.5. The salt contains close to 4 molecules of water of crystallization.

0.1830 gm. air-dried salt lost 0.0238 gm. H_2O at 110° = 12.9 per cent.
Calculated for $C_{20}H_{24}N_2O_2 \cdot C_4H_7O_5N \cdot 4H_2O$ = 13.2 " "

Rotation. c = 1.0 air-dried salt in water; l = 2.2; $\alpha = -2.10°$

$$[\alpha]_D^{20°} = -95.5°$$

l-Anti-Hydroxyaspartic Acid.—The quinine salt above described was dissolved in hot water and most of the quinine precipitated by a slight excess of ammonia. The filtrate was repeatedly extracted with chloroform to remove the remaining alkaloid and then concentrated under diminished pressure to about 15 cc. Dilute nitric acid was then added until the reaction was just acid to Congo red and the free acid crystallized readily. A single recrystallization from hot water gave an optically pure product. The acid crystallizes in wedge-shaped prisms, soluble in about 45 parts of water at room temperature and save for its sign of rotation it has properties identical with those of the dextro acid. For analysis it was dried at 60° over phosphorus pentoxide.

0.1747 gm. substance : 0.2051 gm. CO_2, 0.0781 gm. H_2O.

$C_4H_7O_5N$.	Calculated.	C 32.2,	H 4.70.
	Found.	" 32.0,	" 4.95.

Rotation. c = 2.0 in water; l = 2.2; $\alpha = -0.52°$

$$[\alpha]_D^{20°} = -11.9°$$

Action of Nitrous Acid on d- and l- Anti-Hydroxyaspartic Acids.—In each case 0.5 gm. of the active acid was dissolved in 20 cc. of water together with 0.5 cc. of concentrated hydrochloric acid. Silver nitrite (0.7 gm.) was added by degrees in the course of 24 hours. The mixture was allowed to stand for a further day after which the reaction was complete. Silver chloride was then filtered off and the filtrate concentrated to 15 cc. On examination in a 2.2 dm. tube the solutions were found to be absolutely inactive and on addition of ammonia and calcium acetate a large yield of the characteristic calcium mesotartrate was at once obtained. It was evident that no trace of the active tartaric acids had been produced.

Experiments on the Resolution of Inactive Para-Hydroxyaspartic Acid.

Strychnine Para-Hydroxyaspartate.—3 gm. of the *para* acid suspended in 25 to 30 cc. of water were heated on the water bath with the gradual addition of 6.6 gm. of strychnine. The salt crystallized very readily in regular prisms and was filtered off and recrystallized twice more from 20 cc. of water. The air-dried salt contained 3 molecules of water of crystallization and its specific rotation was constant at $-23.2°$. The yield of thrice crystallized salt was close to 50 per cent.

0.1404 gm. lost 0.0140 gm. H_2O at 110° = 10.0 per cent.
Calculated for $C_{21}H_{22}N_2O_2 \cdot C_4H_7O_5N \cdot 3H_2O$ = 10.1 " "
Rotation. $c = 1.0$ air-dried salt in water; $l = 2.2$; $\alpha = -0.51°$
$$[\alpha]_D^{20°} = -23.2°$$

On decomposing the strychnine salt as already described for the *anti* compound, a good yield of the *para* acid was recovered. It was absolutely inactive in a saturated solution, as was also the mother liquor from which it had crystallized. On treating the mother liquors from the strychnine salt in the same way, the inactive acid was again recovered while the mother liquor which still contained a minute trace of strychnine had an angular rotation of only $-0.04°$ in a 2.2 dm. tube. From these results it is clear that no resolution had been accomplished by the fractional crystallization of the strychnine salt.

Cinchonine Para-Hydroxyaspartate.—The *para* acid (2.5 gm.) and cinchonine (5 gm.) were dissolved in 30 cc. of water. A separation of exceptionally fine clear hard hexagonal prisms readily occurred on cooling. The first crop of crystals (6.0 gm.) was recrystallized twice more from water (20 cc.). The air-dried salt, representing about 45 per cent of the theoretical amount, contains rather more than 2 molecules of water of crystallization.

0.1152 gm. lost 0.0101 gm. H_2O = 8.77 per cent.
Calculated for $C_{19}H_{22}N_2O \cdot C_4H_7O_5N \cdot 2H_2O$ = 7.66 " "
Rotation. c = 1.0 air-dried salt in water; l = 2.0; α = +2.45°
$[\alpha]_D^{20°} = +122.5°$

On decomposing the cinchonine salt as already described, an absolutely inactive *para*-hydroxyaspartic acid was recovered. The mother liquors from the cinchonine salt on similar treatment gave exclusively the inactive acid showing that no resolution had been accomplished.

Brucine Para-Hydroxyaspartate.—This salt which is extremely soluble in water was crystallized from alcohol. The acid (2.5 gm.) was neutralized with brucine (7.5 gm.) in hot aqueous solution which was then evaporated to a syrup and stirred with about 4 volumes of absolute alcohol. The salt separates out readily in fine thin plates containing 4 molecules of water of crystallization. The anhydrous salt is extremely hygroscopic and was dried over phosphorus pentoxide at 110° under reduced pressure.

0.3000 gm. air-dried salt lost 0.0355 gm. H_2O = 11.8 per cent.
Calculated for $C_{23}H_{26}N_2O_4 \cdot C_4H_7O_5N \cdot 4H_2O$ = 11.7 " "
Rotation. c = 2.0 air-dried salt in water; l = 2.2; α = −1.03°
$[\alpha]_D^{20°} = -23.4°$

The recrystallized brucine salt gave on decomposition only the inactive *para* acid and the same was recovered exclusively from the mother liquors, showing that no resolution had been effected.

Quinine Para-Hydroxyaspartate.—This salt readily crystallizes in masses of fine felted needles on warming 2.3 gm. of the acid with quinine (5.8 gm.) in 30 to 40 cc. of water. The salt was crystallized from water four times, but the acid recovered either from the salt or mother liquor was optically inactive. The air-dried salt retains 2 molecules of water of crystallization. The

anhydrous salt obtained by drying under reduced pressure at 120° is very hygroscopic.

0.1537 gm. air-dried salt lost 0.0108 gm. H_2O = 7.02 per cent.
Calculated for $C_{20}H_{24}NO_2 \cdot C_4H_7O_5N \cdot 2H_2O$ = 7.09 " "
Rotation. c = 1.0 air-dried salt in water; l = 2.2; $\alpha = -2.55°$
$$[\alpha]_D^{20°} = -116°$$

Action of Penicillium on Para-Hydroxyaspartic Acid.—A gram of the acid was converted into the monosodium salt by neutralizing with sodium hydroxide, using litmus as indicator. The solution was then diluted to 200 cc. with an inorganic nutrient solution and heavily sown with *Penicillium glaucum.* The solution contained in a beaker was loosely covered with a clock-glass and no special precautions were taken to exclude infection with other organisms, since it appears that in many cases a satisfactory resolution is more often accomplished with mixed cultures than with a single pure organism. An excellent growth of the mold was obtained and at the end of 3 weeks the solution was just acidified with acetic acid, boiled with a little charcoal, and filtered. The filtrate was entirely inactive and on recovering some of the unchanged acid by means of the lead salt, it also was found to be inactive. No resolution had been affected therefore.

Action of Fermenting Yeast on Para-Hydroxyaspartic Acid.—These experiments were made substantially in accord with Felix Ehrlich's (3) excellent method for the resolution of inactive amino-acids. 2 gm. of the *para* acid were converted into the monosodium salt and then mixed with a solution of cane-sugar (50 gm.) in water (500 cc.). 50 gm. of carefully washed yeast were then added and fermentation was allowed to proceed to completion which took 4 to 5 days at 28°. The filtered solution which contained no sugar was then concentrated under reduced pressure and the unchanged acid recovered by precipitation with lead acetate in neutral solution. The lead precipitate was filtered off, well washed, suspended in hot water, and decomposed with hydrogen sulfide. The bulk of the acid recovered, which was about 0.8 gm., was optically inactive and readily crystallized. The mother liquor, however, had a definite rotation of + 0.19° in a 2.2 dm. tube and apparently represented a weak solution (less than 1 per cent) of the dextro and inactive acids On treatment with hydrochloric

acid and silver nitrite the rotation of the solution was not abolished as was the case with the active *anti* acids but was slightly increased (+0.24°). On concentrating the solution and adding potassium acetate, a separation of acid potassium tartrate was easily obtained. On dissolving the washed crystalline tartrate in dilute hydrochloric acid, a distinct dextro-rotation (+0.11°) was observed in a 2 dm. tube and this rotation was increased on addition of Walden's uranium reagent. The results of this experiment, which were confirmed by repetition, made it appear probable that a small amount of resolution of the inactive *para*-hydroxyaspartic acid had been affected and that the levo component was preferentially utilized by the yeast. Furthermore, it appears that the *d*-amino-acid gives *d*-tartaric acid on treatment with nitrous acid. The amount of resolution effected by yeast was insufficient to hold out much hope of isolating the pure active acid by its action.

Addendum.

The Probable Absence of the Hydroxyaspartic Acids in Casein.—The isolation of the two inactive and some of the active forms of hydroxyaspartic acid referred to in the preceding paper made it possible to search more intelligently for this amino-acid among the products of protein hydrolysis. A careful examination of the products from casein has failed to reveal its presence and furnishes additional evidence for the rejection of Skraup's statements to the contrary.[2]

Owing to the relative instability of hydroxyaspartic acid to the prolonged action of acids, the casein was hydrolyzed by tryptic digestion over 5 months. The neutral monoamino-acids were removed by extraction with butyl alcohol and the residue was then precipitated with lead acetate in neutral solution. The lead precipitate was decomposed with sulfuric acid and the filtrate warmed with excess calcium carbonate in order to remove most of the phosphates. The acid calcium salts of hydroxyaspartic acid are fairly soluble and should be formed in the filtrate if present. The filtrate was concentrated to about 200 cc. and the excess of barium hydroxide added with a view to obtaining the insoluble neutral barium salts of hydroxyaspartic acids. The

[2] Compare Dakin, H. D., *J. Biol. Chem.*, 1921, xlviii, 273.

precipitate on decomposition with sulfuric acid was found to contain a little organic phosphorus but very little (0.11 gm.) amino nitrogen. No hydroxyaspartic acid could be induced to crystallize even after "seeding," nor could its phenylisocyanate derivative be obtained. It is concluded that no significant amount of hydroxyaspartic acid is formed by the tryptic digestion of casein.

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THE HYDROGEN ION CONCENTRATION AND BICARBONATE LEVEL OF THE BLOOD IN PNEUMONIA.*

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Certain studies of the acid-base equilibrium in disease were reported from this clinic a year ago (1).

The method employed was in brief the determination of the carbon dioxide dissociation (or absorption) curve, and the carbon dioxide content of the patient's arterial and venous blood, and from these data the construction of the carbon dioxide diagram of Haggard and Henderson (2).

The method was described in the first paper (1). The further studies reported here were carried out in an identical manner, except that the Van Slyke (3) blood gas apparatus was used instead of the Henderson (4). The combined method of Van Slyke and Stadie (5) was used. In the present research all arterial or A-points, and all venous or V-points, were placed on the dissociation curve at its intersection with the abscissa representing the carbon dioxide content of the blood as found by analysis. All the curves obtained are shown in Figs. 1 to 7. They are all for blood equilibrated with air and various tensions of carbon dioxide at the patient's body temperature.

Since in venous blood and in some pathologic arterial bloods there is an oxygen unsaturation, the curve obtained for fully aerated blood does not truly represent that of the blood as it exists in artery or vein. The effect of oxygen unsaturation according to

* This paper is No. 26 of a series of papers on the physiology and pathology of the blood from the Harvard Medical School and allied hospitals, a part of the expense of which has been defrayed by a grant from the Proctor Fund for the study of chronic disease. A parallel and more extensive study of the acid-base balance in pneumonia has been carried out at the Boston City Hospital by Drs. Buckman, Adams, and Edwards. These results will be published shortly as a part of this series of papers.

Haldane and his coworkers (6) and to Peters, Barr, and Rule (7) is to shift the curve upwards. According to Haggard and Henderson (8) this phenomenon does not take place in oxalated blood. The blood used in this research was oxalated, but since Henderson and Haggard's findings have not been confirmed by others we have here plotted the A- and V-points on the curves for fully oxygenated blood but have also calculated the possible effect of oxygen unsaturation by the formula of Peters, Barr, and Rule (7). The position of the points when so corrected is shown in the several figures by circles containing crosses, the uncorrected points by plain circles.

As has been shown by Haggard and Henderson, the position of A- or V-points with respect to a series of radii drawn through the zero point gives the hydrogen ion concentration of the blood. In the figures in this paper the diagonals for various hydrogen ion concentrations (pH) have been constructed as in the paper of Peters, Barr, and Rule (7), and in Table I is given the pH for the various points, both corrected for oxygen unsaturation, and uncorrected, as read off from these diagonals. The diagonals so constructed, however, are for a temperature of 38°C. Since the solubility of carbon dioxide in blood is one of the factors used in the construction of the pH diagonal, and since the solubility varies with the temperature, the position of the diagonal will also vary with the temperature. The solubility coefficient of carbon dioxide in whole blood according to Bohr (9) is 0.937 at 15°C. and 760 mm., and is 0.511 at 38°C. and 760 mm. If the temperature solubility coefficient curve is a straight line (which may legitimately be assumed for the present purpose), then the solubility coefficient at 40°C. and 760 mm. would be 0.474. Using this coefficient for the construction of a given pH line, we find that what it amounts to is that at 40° a given diagonal represents a pH that is lower by 0.04 than that at 38°. This correction has been made in the table by adding 0.01 to the pH as read from the diagonals for each half degree of temperature elevation above 38°. In the figures it is shown by moving the A- and V-points in relation to 38° diagonals, this being more satisfactory than drawing complete sets of diagonals for each patient's temperature. The points so corrected are shown by black dots. These black dots then probably represent the true pH of the blood as

nearly as we can measure it by this method, since they are corrected both for oxygen unsaturation and for body temperature. The temperature correction is an approximation; it is probably maximal and sufficiently near the truth for the present purpose.

The studies presented in this paper deal entirely with the blood of pneumonia patients. In the previous paper (1) the diagrams for the bloods of three pneumonia patients were shown. We felt at that time that there was some evidence, from the position of the A-points, of an increase in the hydrogen ion concentration of the blood of these patients. Peters has criticized this conclusion on the basis that the effect of oxygen unsaturation was not taken into account and that, if it were, the hydrogen ion concentration of the three bloods under discussion would be found quite normal. Whether this is a valid criticism or not will depend on who proves correct in the controversy over the effect of oxygen unsaturation on oxalated blood. We will not enter this controversy now. The object of the present research was by further study to try to discover whether any change in hydrogen ion concentration or level of blood bicarbonate occurs in pneumonia. In our figures and table the pH, uncorrected, corrected for oxygen unsaturation, and corrected for both oxygen unsaturation and body temperature may all be found.

In this communication we will present for discussion seventeen carbon dioxide diagrams of the bloods of ten pneumonia patients. These patients were also made the subject of a study of the effect of oxygen therapy. Their histories have been reported in full in that connection by Barach and Woodwell (10), so that it will be unnecessary to repeat them here; the case numbers used here are the same as those used by Barach and Woodwell.

All data except the actual curves will be found in Table I, the curves themselves in Figs. 1 to 7. Ten of the seventeen curves were obtained during the height of the disease and before the patients had received any alkali or oxygen therapy. The remaining seven were taken either after crisis or after treatment.

The Blood pH in Pneumonia.

The matter of the state of the acid-base balance in pneumonia may for convenience be separated into the related matters of blood reaction as indicated by hydrogen ion concentration (pH)

TABLE I—Blood Gas

Case No.*	Date.	Diagnosis.	Day of disease.	Temperature.	Oxygen saturation.		Carbon dioxide content.		Carbon dioxide tension.		pH uncorrected.	
					A†	V†	A	V	A	V	A	V
	1920			°C.	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*mm.*	*mm.*		
11	Nov. 22	Lobar pneumonia.	3rd	38.5	98.8	66.0	53.5	56.3	37.0	44.0	7.41	7.36
14	" 30	" "	7th	39.5	74.4	54.8	47.0	46.4	51.0	49.5	7.21	7.21
	Dec. 2		9th	38.5	91.6	36.0	47.2	53.7	36.0	71.5	7.36	7.11
22	" 11	Bronchopneumonia. Septicemia.	7th	40.2	91.9		37.8		40.5		7.21	
23	" 13	Lobar pneumonia.	4th	40.2	78.6	65.9	51.6	52.8	51.5	54.0	7.24	7.23
	1921											
17	Jan. 4	Bronchopneumonia. Septicemia.	21st	39.5	93.9	77.1	42.4	43.8	39.0	42.0	7.27	7.26
	" 5		22nd	39.0	97.7	85.2	44.5	46.5	24.5	29.0	7.51	7.46
13	" 5	Lobar pneumonia.	5th	40.3	92.4		42.0		41.5		7.25	
	" 18			36.4	96.5		50.6		45.0		7.29	
20	" 28	Lobar pneumonia.	7th	40.0	92.5	64.1	36.6	41.4	45.5	59.5	7.15	7.07
	Feb. 15			37.0	96.2		55.0		44.0		7.35	
10	Jan. 31	Lobar pneumonia. Pulmonary tuberculosis.	6th	39.1	77.2	50.2	49.5	53.1	46.0	53.0	7.27	7.23
	Feb. 2		8th	39.1	62.3	53.5	60.4	60.2	90.0‡	90.0‡	7.05	7.05
	" 3		9th	39.1	82.2	78.4	66.7	70.0	60.5	67.5	7.28	7.26
21	Mar. 12	Lobar pneumonia.	14th	38.9	81.1		45.6		46.5		7.23	
	" 14			37.9	88.5		48.1		44.5		7.27	
19	" 16	Lobar pneumonia. Pulmonary tuberculosis.	11th	39.0	87.5	78.6	40.4	41.2	30.0	31.5	7.38	7.37

* Case Nos. are the same as in Barach and Woodwell (10).
† A = Arterial; V = Venous.
‡ Approximate only.

Data in Pneumonia.

pH corrected for oxygen unsaturation.		pH corrected for oxygen unsaturation and for body temperature.		Remarks.
A	V	A	V	
7.41	7.41	7.42	7.42	Right lower lobe solid. Rest of chest fairly dry. Moderate dyspnea. No cyanosis. Recovered by crisis on 7th day.
7.24	7.28	7.27	7.31	Very sick, pneumonia (both lower lobes). Marked dyspnea. Marked cyanosis. Very poor quality pulse.
7.39	7.32	7.40	7.33	Still very sick. A little less dyspnea and cyanosis. Later developed empyema and died.
7.23		7.27		Nearly moribund. Deep cyanosis (stagnant type of anoxemia). Rapid shallow breathing. Rapid feeble pulse. Died 2 hours after observation.
7.26	7.28	7.30	7.32	Very sick. Delirious. Moderate cyanosis and dyspnea. Died 6 hours after observation.
7.27	7.29	7.30	7.32	Very sick. Stuporous. Extreme hyperpnea. No cyanosis. Consolidation of left base, rest of chest dry.
7.51	7.50	7.53	7.52	Since yesterday given 30 gm. sodium bicarbonate with some relief to respiratory distress. 5 days later developed erysipelas and died.
7.26		7.30		Fairly comfortable. Slight cyanosis. Moderate dyspnea. Consolidation of right lower lobe. Râles at left base.
7.29		7.26		Had crisis on 9th day of disease. Now convalescent. 18 days since onset.
7.16	7.13	7.20	7.17	Moderately ill. No great respiratory distress. Slight cyanosis. Consolidation of right upper lobe, rest of chest clear.
7.35		7.33		Recovered by crisis on 8th day of disease. Now well. Today is 25th day since onset.
7.31	7.30	7.33	7.32	Very sick. Moderate dyspnea. Marked cyanosis. Râles in whole left chest. Consolidation of left base and below right clavicle.
7.14	7.15	7.16	7.17	Seems in extremis. Outspoken generalized pulmonary edema.
7.30	7.28	7.32	7.30	Better today. Improvement followed oxygen and alkali therapy. Later recovered from his pneumonia, but at home died of phthisis.
7.24		7.26		Severely ill. Moderate dyspnea. Slight cyanosis.
7.28		7.28		Crisis yesterday (15th day of disease). Very comfortable today. Slight dyspnea still. Later developed empyema. Operated. Recovered.
7.40	7.40	7.42	7.42	Marked prostration. Moderate cyanosis and dyspnea. Consolidation of both lower lobes. Rest of chest clear. Later developed phthisis.

Case No.*	Date.	Diagnosis.	Day of disease.	Temperature.	Oxygen saturation.		Carbon dioxide content.		Carbon dioxide tension.		pH uncorrected.	
					A†	V†	A	V	A	V	A	V
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pH corrected for oxygen unsaturation. A	pH corrected for oxygen unsaturation. V	pH corrected for oxygen unsaturation and for body temperature. A	pH corrected for oxygen unsaturation and for body temperature. V	Remarks.
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and the level of blood alkali. The former is shown by the position of the A- and V-points.

The pH of normal arterial blood as well as one can judge by the literature is not far from 7.35. Peters, Barr, and Rule (7) got this average for fully oxygenated blood of normal persons; their maximum was 7.42 and minimum 7.29. Roughly then, a pH between 7.30 and 7.40 may be considered normal. Now the average arterial pH of our ten sick pneumonia patients was as follows:

	pH
Uncorrected	7.26
Corrected for oxygen unsaturation	7.28
Corrected for oxygen unsaturation and for body temperature.	7.31

The range of variation is somewhat greater than in normal arterial blood, the maximum being (in the case of the doubly corrected values) 7.42 and the minimum 7.20. It would seem then on the basis of the corrected values for pH that there is no constant tendency toward an abnormal hydrogen ion concentration of the arterial blood in pneumonia. The average figure of 7.31 cannot be regarded as outside the normal range. Certain individual cases, however, seem to present figures somewhat lower than do any normals; for example, Nos. 14, 21, 22, and 20, with pH of 7.27, 7.26, 7.27, and 7.20 respectively. These results seem to indicate that in certain cases of pneumonia the hydrogen ion concentration actually is shifted in the direction of decreased alkalinity.

Bicarbonate Level.

The matter of the level of blood alkali is shown by the level of the dissociation curves. The extreme variations in the levels of the curves of normal persons are not known. In the previous paper a zone was shown within which all normal curves found in the literature (except those of Straub and Meier, 11) fell. This zone is shown again in the various figures of the present paper.

Peters, Barr, and Rule (7) show a very similar zone. Their lower border is essentially the same as ours, the upper border a little higher in order to contain the rather high level curves of the subject J. P. The levels of curves of pathologic blood in relation to the normal zone can be seen in the various figures. To

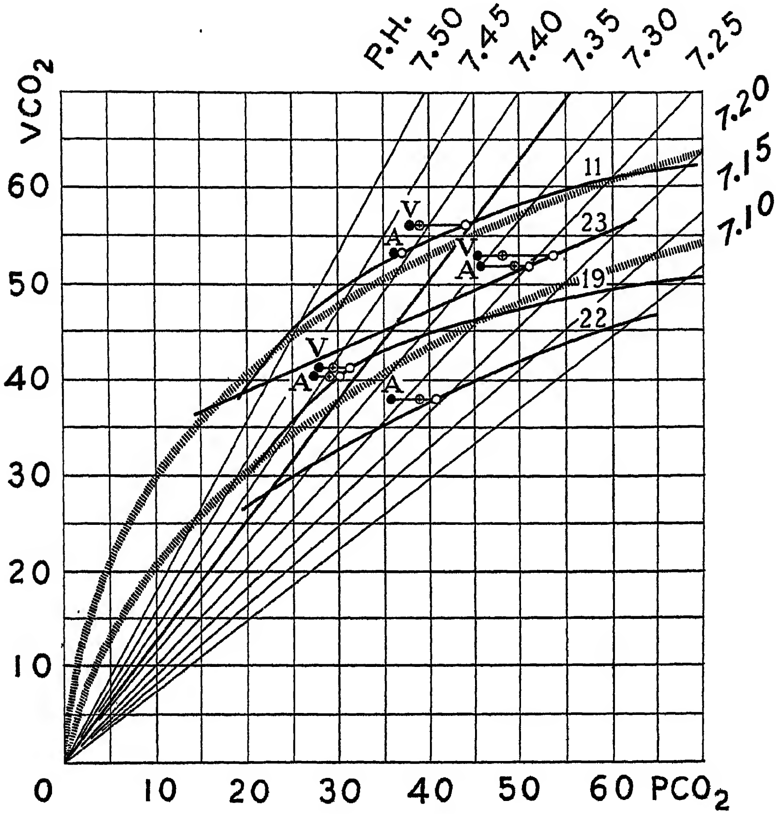

FIG. 1. Carbon dioxide diagrams in Cases 11, 19, and 23 (lobar pneumonia), and in No. 22 (bronchopneumonia).

In this and in all following figures the arterial points, A, and the venous points, V, are shown by circles. The points corrected for oxygen unsaturation are shown by circles containing crosses, and those corrected for body temperature as well by black dots.

The shaded zone indicates the area within which we believe the curves of normal blood should fall. P CO_2 = pressure of carbon dioxide in mm. of mercury. V CO_2 = carbon dioxide content of the blood in volumes per cent.

This figure shows three cases of lobar pneumonia with essentially normal diagrams, and one of bronchopneumonia with a slight depression in curve level and slight reduction in arterial pH.

For further data on these cases and also on those in following figures see Table I of this paper and also the paper of Barach and Woodwell (10).

measure actually the height of the curves we can read off the carbon dioxide content either with respect to isohydric points along, let us say, the pH 7.35 diagonal, or perhaps more conveniently for points of equal carbon dioxide tension, as for example along the 40 mm. ordinate. Peters, Barr, and Rule (7) found that at 40 mm. tension the average carbon dioxide content of all normal subjects of their own and from the literature (except those of Straub and Meier which they excluded for the same reasons as did Means, Bock, and Woodwell) was 49.3 volumes per cent, the maximum 55.9 and the minimum 43.3. The average carbon dioxide content of our ten sick pneumonia patients at 40 mm. carbon dioxide tension was 43.2 volumes per cent with a maximum of 54.5 and a minimum of 35.0. The pneumonia curves, therefore, show a lower average level than the normals, but also a greater range of variation. Half of them are essentially within normal limits, half somewhat lower than normal. From this we should conclude that in pneumonia there might either be a normal level of blood alkali or a slightly reduced one. A low level of blood alkali is according to Henderson's views (2) capable of one of two explanations, first as being due to non-volatile acidosis, second to acapnia. The latter is a condition of blood reaction more alkaline than normal, never of less alkaline; but our pneumonia diagrams show either a normal pH or one shifted in the direction of less alkalinity, hence those which show a low level curve must denote non-volatile acidosis rather than acapnia. The non-volatile acidosis when it occurs is slight; it is nothing like the marked lowering of blood alkali seen in diabetic or renal acidosis (see curves in first paper, 1).

To summarize our conclusions to this point we should say that the series of pneumonia bloods as a whole suggests that there may be at the height of this disease either a normal pH and level of blood alkali or that on the other hand in certain cases there may be an acidosis. This acidosis may be an actual non-volatile acidosis (but of slight degree only) as shown by a dissociation curve below the normal level, or it may be simply a carbon dioxide acidosis, that is to say no lowering of the dissociation curve but a change in blood reaction, as shown by the position of the A-point in the acid direction. This latter suggests that sometimes there may be insufficient pulmonary ventilation with a resulting re-

tention of carbon dioxide in the blood to an abnormal concentration. The two forms of acidosis may, theoretically at least, coexist.

The attempt was made to see whether the acid-base data furnished any clue to prognosis. None could be found except that all the fatal cases showed a pH of not over 7.30; but against this must be put the recovery of Case 20 who had the most marked acidosis of all, pH 7.20. Between carbon dioxide capacity at 40 mm. and death or recovery, there was no relation at all.

We also looked for possible relationships between pH and arterial oxygen saturation, and between carbon dioxide capacity at 40 mm. and arterial oxygen saturation, and again found none.

A further study of the individual cases brings out one or two points which may perhaps be of interest. In the first place the situation in pneumonia, if our interpretation of the findings is correct, is that when abnormalities exist they are acidoses, either non-volatile or carbon dioxide, or both. An appreciation of this situation introduces one or two clear-cut indications for treatment. We have recently discussed these indications in their broader aspects elsewhere (12). It will suffice here to point out that an individual suffering from respiratory embarrassment may theoretically be helped by having the level of his dissociation curve raised. This is true whether the curve is at a low level to start with or at a normal level but with a lowered pH. Raising a curve with a lowered pH may render the pH normal without change in carbon dioxide tension. This will mean that an insufficient pulmonary ventilation becomes efficient without increasing in volume. In other words, as brought out by Henderson and Haggard (13), it requires less ventilation to maintain normal hydrogen ion concentration at a given rate of carbon dioxide excretion with a high level of the dissociation curve than with a low one. To our minds, with two possible objections which we will discuss presently, it would seem desirable for the pneumonia patient with respiratory embarrassment to have the level of his dissociation curve raised.

Effect of Alkali Administration.

That the dissociation curve can be raised by the administration of sodium bicarbonate has been proved—the case of the nephritic

for example in the previous paper (1) or of Case 10 in the present (Fig. 7), and experimentally by Haggard and Henderson (2).

The two objections to alkali therapy which have been raised are these: In the first place, if, as the curve rose in level as alkali was given, the A-point passed to the left of the pH 7.35 diagonal, which it would do if a compensatory fall in pulmonary ventilation did not occur, we should be producing an alkalosis which in itself might be harmful. The second objection is one raised by Peters when we first reported this work, and that is that raising the curve and diminishing the pulmonary ventilation in pneumonia might be harmful because it would produce or increase an existing anoxemia.

Whether the phenomenon mentioned in the first objection occurs with any regularity we do not know. It did happen to a certain extent in the nephritic already referred to and in Case 17 of the present paper (Fig. 6), in neither instance, however, with any apparent ill effects that could be attributed to alkalosis. In Case 10, however, a marked rise in the curve occurred with no alkalosis developing. It seems to us that the likelihood of producing a dangerous alkalosis is slight, particularly if the reaction of the urine is carefully followed and alkali administration stopped at once upon its becoming alkaline.

Peters' objection is a perfectly valid one. Anoxemia does often exist in pneumonia. Decreasing pulmonary ventilation might aggravate it. Giving alkali to diminish pulmonary ventilation, therefore, might be the diametrically wrong thing to do in pneumonia. The answer to the objection is, of course, that we should not only raise the dissociation curve by alkali administration but at the same time abolish anoxemia by oxygen administration. That anoxemia in pneumonia can be relieved or abolished by oxygen therapy has been proved in this clinic (10) and by others (14).

Arterial and Venous Blood in Pneumonia.

Another matter on which the present studies may throw some light is that of the relation between the reaction of arterial and venous blood in pneumonia. Peters, Barr, and Rule have already discussed this relationship in the bloods of normal persons. They state their findings in three normal subjects as follows:

"The CO_2 tension of venous blood was found to vary between 42 and 72 mm. uncorrected for oxygen unsaturation, 39.5 to 58.5 mm. after correction, with an average of 50.2 mm. The corresponding values for pH were 7.37 to 7.12 uncorrected, 7.40 to 7.22 corrected, with an average of 7.31."

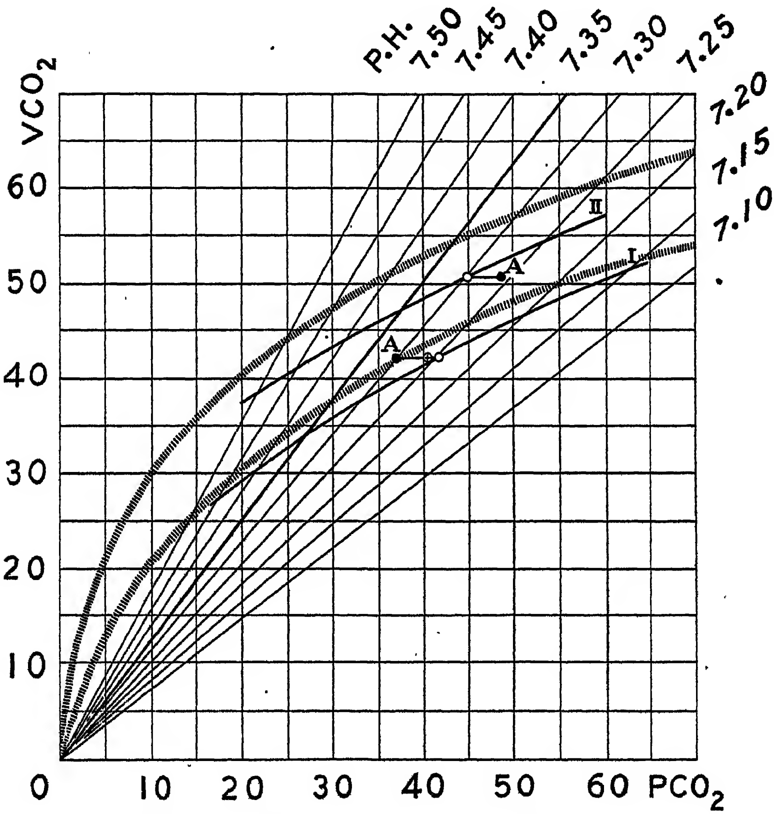

FIG. 2. Carbon dioxide diagrams in Case 13 (lobar pneumonia), I, on the 5th day of the disease and II, 9 days after the crisis, which was on the 9th day of the disease.

In this case a curve just below the normal limit assumes a normal level after the crisis.

The effect of oxygen saturation on carbon dioxide-combining power is exerted in the direction of keeping blood reaction nearly constant, of their three normals the average corrected arterial

and venous pH both being 7.31. Taking out of our ten sick and untreated pneumonia cases the seven in which we have observations on both arterial and venous blood, we find an average pH corrected for oxygen unsaturation of 7.29 for both arterial and venous blood, and corrected for body temperature 7.32. Even in the four most acidotic of these bloods we find an average arterial pH, corrected for oxygen unsaturation and body temperature, of 7.27, and for the venous 7.28. In pneumonia then (even in the presence of acidosis) there is, as there is in normal persons, little if any difference in pH between arterial and venous blood.

Effect of the Crisis.

In considering those cases of our series in which we have more than one curve, certain interesting features appear. In three cases for example (Nos. 13, 20, and 21) we have observations before and after the crisis. In each of these the second observation showed a higher level of the curve than the first, thus:

Case No.	CO_2 content at 40 mm.		Rise in level of curve.
	Before crisis.	After crisis.	
	vol. per cent	*vol. per cent*	*vol. per cent*
13	41.3	48.3	7.0
20	35.0	53.2	18.2
21	41.3	45.3	4.0
Average..			9.7

The pH of the arterial blood (corrected for oxygen unsaturation and for body temperature) of these same patients before and after crisis was as follows:

Case No.	Arterial pH.	
	Before crisis.	After crisis.
13	7.30	7.26
20	7.20	7.33
21	7.26	7.28
Average.............	7.28	7.29

In these three cases therefore, there was seen following a crisis a return of the blood acid-base balance toward normal in two directions. The available alkali as shown by the curve levels lower than normal before crisis in all three (slightly in Nos. 13

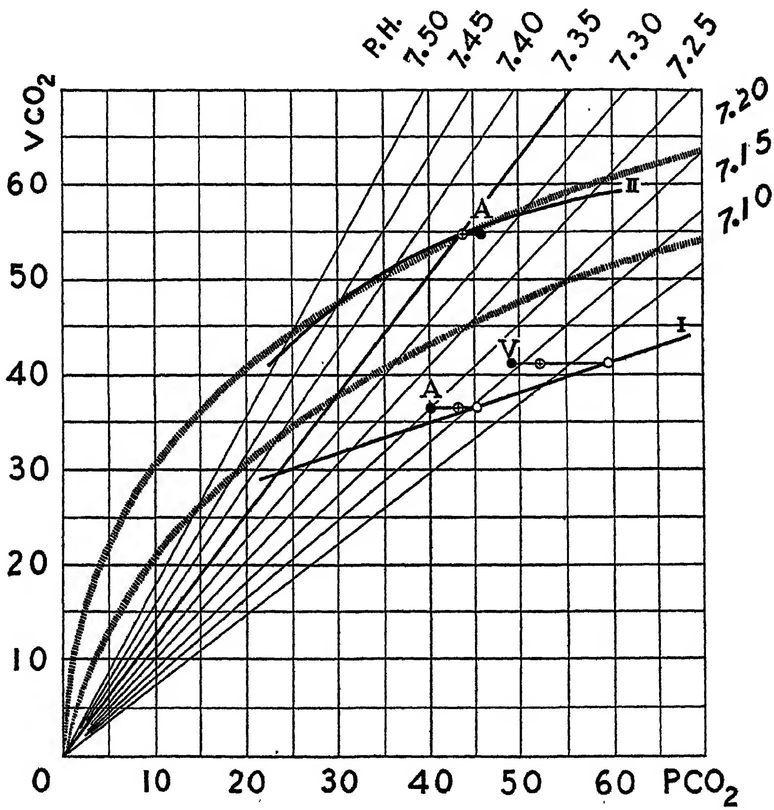

FIG. 3. Carbon dioxide diagrams in Case 20 (lobar pneumonia), I, on the 7th day of the disease and II, 17 days after the crisis, which was on the 8th day of the disease.

This case showed a marked rise in curve level from a low position to a high normal position after the crisis and a rise in pH from 7.20 to 7.33.

and 21 and definitely in No. 20) in each instance rose to an entirely normal level after the crisis. This is most marked in Case 20, which is not surprising as this patient had the most acidosis to start with and also in this case the second observation

was 17 days after the crisis, while in No. 13 the second observaions was only 9 days after crisis and in No. 21 only 1 day.

The hydrogen ion concentration in two of the three cases showed a tendency to move from a less alkaline reaction than normal

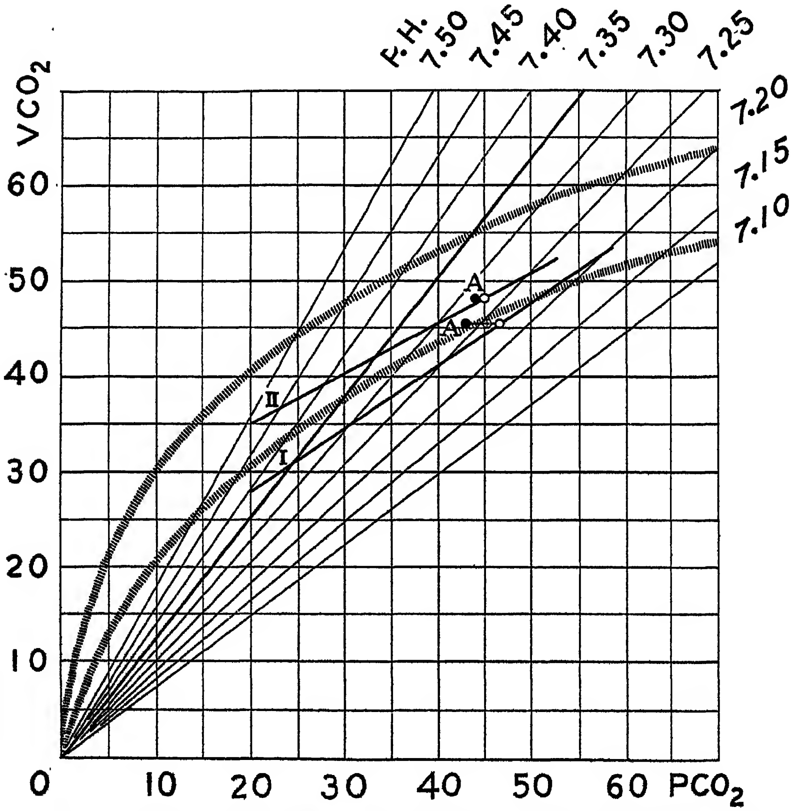

FIG. 4. Carbon dioxide diagrams in Case 21 (lobar pneumonia), I, day before crisis, II, day after crisis. The crisis was on the 15th day of the disease.

This case shows a slight rise in curve level the day after the crisis.

toward a normal one after crisis. This phenomenon like the other is more noticeable in Case 20, the one most acidotic to start with.

The changes undergone by the blood in these three cases before and after the crisis are shown diagramatically in Figs. 2, 3, and 4.

The effect on the acid-base balance of the two therapeutic measures which as suggested above and elsewhere (12), seem to us on theoretical grounds often indicated, either alone or coin-

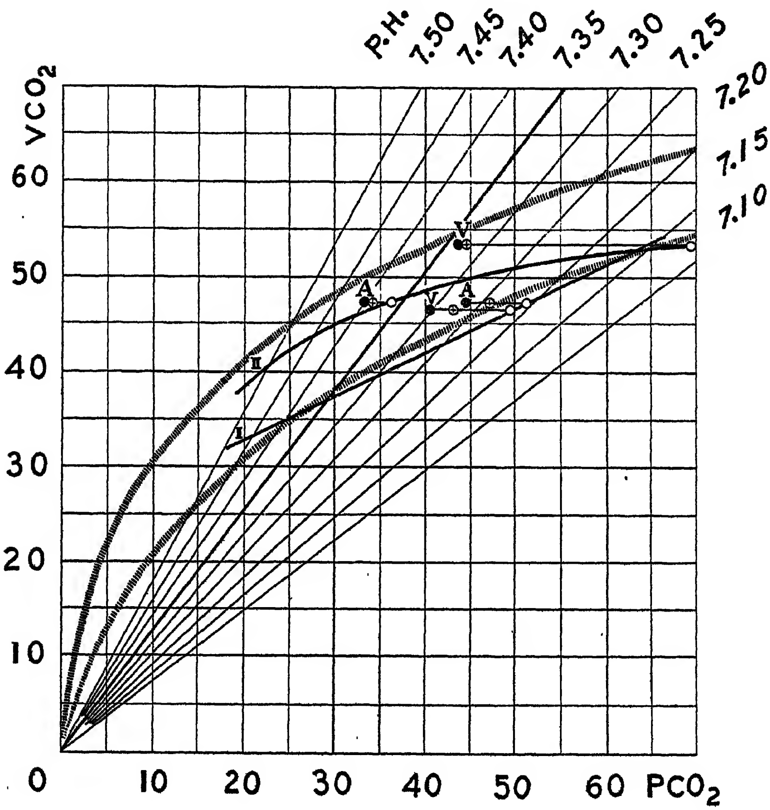

Fig. 5. Carbon dioxide diagrams in Case 14 (lobar pneumonia), I, on 7th day and II, on 9th day of disease. Between Curves I and II he was treated intensively with oxygen.

Here a slight rise in curve level and pH followed oxygen therapy but with no crisis.

cidently, namely alkali and oxygen therapy, also receive some light from the present research.

The outstanding effect of oxygen therapy is, of course, relief of anoxemia. This has been discussed in detail in other papers

(10, 12), but it is theoretically at least conceivable that relief of anoxemia may have in itself some beneficial effect upon acidosis. The recovery of normal blood alkali level just described, which

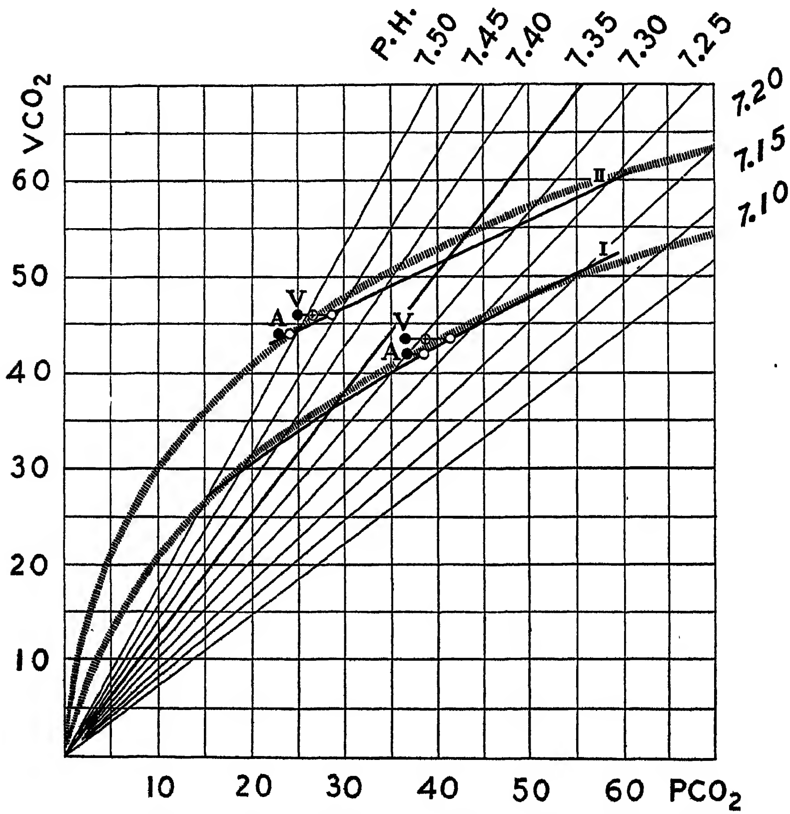

FIG. 6. Carbon dioxide diagrams in Case 17 (bronchopneumonia), I, on the 21st and II, on the 22d day of the disease. Between the two he received 30 gm. of sodium bicarbonate.

In this case a rise in curve level followed alkali administration and the pH was shifted to the alkaline side of normal, an alkalosis was produced but no harmful signs or symptoms of that condition appeared.

occurs after crisis, could in the three cases under discussion hardly have been due to oxygen therapy for no one of these received oxygen except for a short period. Case 14, however, in which we have two sets of data 2 days apart, showed a rise in curve level

and return of blood reaction from an acidotic to a normal one although he had had no crisis and on the second observation was still very ill though less dyspneic. He eventually died of empyema. The results in this case are shown in Fig. 5. From the

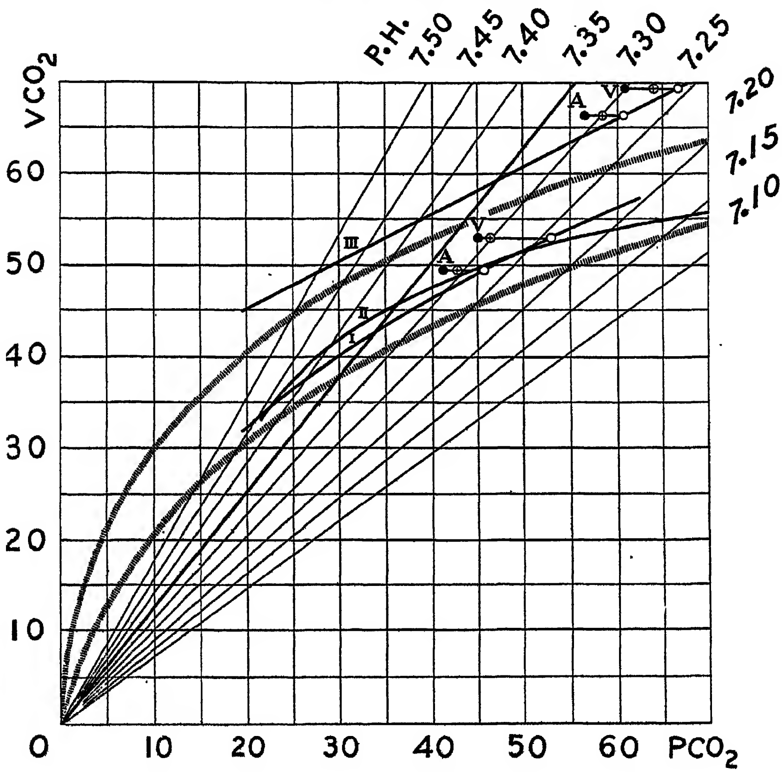

FIG. 7. Carbon dioxide diagrams in Case 10 (lobar pneumonia), I, on the 6th, II, on the 8th, and III, on the 9th day of the disease. Between Curves II and III he was treated with oxygen and was given 15 gm. of sodium bicarbonate.

A marked rise in curve level without change in pH resulted.

time of the first curve on the 7th day of the disease to that of the second curve on the 9th day, the arterial oxygen saturation rose from 74.4 to 91.6 per cent, due, it was believed, to the vigorous oxygen therapy which he received. As to whether the rise in

curve level from 41.8 volumes per cent at 40 mm. to 48.5, and that of corrected arterial pH from 7.27 to 7.40, were in any way the result of oxygen therapy one can only speculate. The problem, however, in that respect is worthy of further study.

The effect of alkali administration was studied in two cases. In Case 17 (Fig. 6), between the first and second curves 30 gm. of sodium bicarbonate were given by mouth. Probably as a result of this, for there was no crisis, the level of the curve rose from 42.5 volumes per cent at 40 mm. to 51.3, and the corrected arterial pH from 7.30 to 7.53. In this case an actual alkalosis was produced, but no harmful effects due to that were noted and the dyspnea which had been present at the start was somewhat relieved.

In Case 10, shown diagrammatically in Fig. 7, three curves were obtained. The first of these on the 6th day of the disease showed a curve at a normal level and perhaps a very slight carbon dioxide acidosis. On the 8th day of the disease the curve was essentially the same as to level. No satisfactory A-point was obtained. Between the time Curve II was obtained and the next day when Curve III was obtained he was treated intensively with oxygen and was also given 15 gm. of sodium bicarbonate, the dissociation curve showed a rise of 8 volumes per cent at 40 mm. and at the same time some relief in respiratory distress.

SUMMARY.

1. Carbon dioxide diagrams of the bloods of ten new cases of pneumonia are presented. In three cases observations were secured before and after the crisis, in one case before and after oxygen therapy, and in two cases before and after the administration of sodium bicarbonate.

2. The alkali of the blood in pneumonia as shown by the level of the carbon dioxide dissociation curve, that is to say by the carbon dioxide capacity at a fixed carbon dioxide tension (40 mm.), was found to be sometimes within normal limits, sometimes somewhat below normal limits. The average in the pneumonia group was 43.2 volumes per cent, while in normal persons Peters, Barr, and Rule found the average 49.3 volumes per cent. The lowest observed in pneumonia was 35.0 volumes per cent.

3. The arterial pH in pneumonia as calculated from the carbon dioxide diagram and corrected for oxygen unsaturation and body temperature showed an average of 7.31. Four of the ten bloods showed a pH below 7.30, which probably can be considered the lower border of normal variation. The lowest observed was 7.20.

4. No relation between pH or dissociation curve level and degree of anoxemia or prognosis could be found.

5. In pneumonia patients, as in normal persons, there seems to be little or no difference in pH between arterial and venous blood.

6. In three patients studied before and after crisis there was an increase in curve level after crisis in each instance, and in one showing a marked shift in pH before crisis there was a normal pH after crisis. A rise in curve level and a return to a normal pH in the cases with lowered pH would seem to be among the phenomena that take place at or after the crisis.

7. The same phenomena occurred in one case without crisis but after vigorous treatment with oxygen.

8. In two cases the level of the curve was raised apparently by the administration of sodium bicarbonate, in one instance with the production of a slight alkalosis.

9. It is suggested that in pneumonia patients showing acidosis either in the sense of a low level of available blood alkali or of decrease in pH or combination of the two, the administration of sodium bicarbonate may be helpful by diminishing the work of the respiratory bellows. By such a procedure a pH less alkaline than normal may be brought to normal with no increase in ventilation because of a raising in the level of the dissociation curve. Or in a case with low curve but normal pH to start with, the raising of the curve may diminish the amount of ventilation necessary. The use of sodium bicarbonate should be carefully controlled, however, to avoid the production of alkalosis, and when anoxemia is present should be combined with oxygen therapy.

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ANALYSIS AND COMPOSITION OF CORN POLLEN.

PRELIMINARY REPORT.

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INTRODUCTION.

The literature dealing with corn and corn products is very extensive but we have failed to find in it any reference to the composition of corn pollen. In the process of fertilization and reproduction pollen plays a very important part. It would seem, therefore, that some knowledge of the kind and nature of the chemical compounds occurring in pollen would be of interest to plant physiologists. Different varieties of corn apparently produce pollen which varies greatly in composition. This fact might be of importance in cross-breeding.

The present investigation was undertaken in order to contribute some information on the following points: (1) The approximate composition of corn pollen, (2) the principal inorganic constituents of the ash, and (3) the principal organic compounds contained in the pollen grains.

Unfortunately, stress of other work has prevented us from completing the investigation, but we wish to publish this preliminary report giving the results which have been obtained up to the present time because our joint work will be interrupted during the coming year.

EXPERIMENTAL.

The pollen was gathered in the following manner: Corn tassels were cut off as the pollen sacs were opening. The tassels were spread out in thin layers on clean paper on the floor and allowed to dry. The pollen was then shaken out on clean paper and care-

fully sifted. Many of the pollen sacs did not open and in order to obtain the enclosed pollen it was necessary to break them by rubbing or by passing the tassels through a coarse mill and then carefully sifting out the pollen. Practically all foreign matter was finally removed by sifting through very fine bolting cloth.

It is important that fresh pollen be spread out in thin layers while drying as otherwise it will undergo very rapid decomposition. One day about 100 gm. of ripe pollen obtained by shaking fresh corn tassels over paper were left over night in a dish which was loosely covered by a watch-glass. The pollen was a heavy yellow powder which appeared to be dry. The next morning it was found to consist of a semifluid gummy mass in which it was impossible to distinguish any individual pollen grains. We were unable at that time to make any investigation of the changes which had occurred and this observation is merely recorded to indicate the rapidity with which pollen may undergo spontaneous decomposition when conditions of temperature and moisture are favorable.

In 1919 we obtained about 800 gm. of pollen from yellow dent Improved Leaming corn, and in 1920 some 2,000 gm. of pollen were gathered from White Flint Luces Favorite and a smaller quantity from pop-corn.

The pollen obtained as outlined above formed a golden yellow, dense powder and had a strong but agreeable aromatic odor. 100 gm. of pollen occupied about 150 cc. of space.

Determination of Moisture.

On drying at 103°C. the pollen continued to lose in weight slowly and after 48 hours the loss amounted to about 6.5 per cent. All of this loss in weight was not due to loss of water because the dried pollen was dark brown in color and had lost practically all of the characteristic odor. After drying at 100°C. the color darkened and most of the odor was lost. It is evident, therefore, that in drying at these temperatures certain volatile constituents are lost and that some oxidation occurs.

In order to obviate such losses of volatile principles and to prevent oxidation the pollen was dried for analysis at room temperature in vacuum over sulfuric acid. The loss in weight on drying in this manner was 4.68 per cent and there was no noticeable change either in color or odor.

Extraction of the Pollen with Various Solvents.

A series of extractions was made to determine the amounts of material removed from the pollen by ether and alcohol during varying lengths of time. The results obtained are given in condensed form in Table I. The percentages are all calculated to the original air-dry pollen.

TABLE I.

Ether and Alcohol Extraction of Corn Pollen.

Ether extraction; Soxhlet method.				Alcohol extraction following ether extraction.				Amount of alcohol extract soluble in ether.
Extraction.	Pollen used.	Weight of extract.	Percentage of extract.	Extraction.	Pollen used.	Weight of extract.	Percentage of extract.	
hrs.	*gm.*	*gm.*	*per cent*	*hrs.*	*gm.*	*gm.*	*per cent*	
8	5	0.0538	1.07	4*	5	1.6166	32.33	14.93
24	10	0.1320	1.32	24 (Soxhlet)	10	1.1405	11.40	
48	20	0.3170	1.58	168 (Soxhlet)	20	2.9272	14.63	
48	2	0.0280	1.40	4 weeks.†	20	6.8267	34.13	
48	2	0.0285	1.42					

* 5 gm. of pollen were suspended in 75 cc. of absolute alcohol and boiled under reflux condenser for 1 hour on the water bath. The alcoholic solution was decanted and replaced by fresh alcohol which was in turn boiled for 1 hour. These operations were repeated four times. The alcoholic extracts were united, filtered, and evaporated to dryness and then dried in vacuum over sulfuric acid.

† After extracting 20 gm. of pollen with absolute alcohol in a Soxhlet apparatus for 7 days, the pollen residue was suspended in about 100 cc. of absolute alcohol and boiled on the water bath under reflux condenser. The alcohol was decanted and renewed daily until all of the coloring matter was removed and the alcohol remained practically colorless. The time required was 4 weeks. The alcoholic extracts were united, the alcohol was distilled off, and the extract was dried in vacuum over sulfuric acid.

It is interesting to note that extracting pollen with four portions of boiling absolute alcohol yields almost as high a percentage of extract as was obtained after completely exhausting the pollen with absolute alcohol during a period of 4 weeks.

A more complete extraction was obtained when the pollen was suspended in the alcohol in a small flask and digested under a reflux condenser on the water bath than when the pollen was contained in a thimble as in the usual Soxhlet method.

The dried ether extract was of a dirty green color and of a rather soft, wax-like consistency. This extract contained only a trace of phosphorus and consequently it could only contain a very small amount of phosphatide. The nature of this fat or wax-like material has not been determined.

Extraction of Corn Pollen with Absolute Alcohol and Chloroform.

It has been stated by Glikin (1) that the method of Rosenfeld (2) gave high yields of fat and lecithin, particularly in the analysis of animal tissues. This method consists in extracting the material for ½ hour in boiling alcohol and then extracting the residue for 6 hours with chloroform in a Soxhlet apparatus.

We employed this method on corn pollen as follows: (*a*) 5 gm. of pollen after drying in vacuum over sulfuric acid, were placed in an extraction thimble and extracted for 2 hours by immersing in about 50 cc. of boiling absolute alcohol contained in a large test-tube. The thimble and contents were then rinsed with absolute alcohol. The solution was filtered, the alcohol evaporated, and the extract dried to constant weight in vacuum over sulfuric acid. The dry extract weighed 0.3815 gm. or 7.63 per cent of the air-dried pollen. (*b*) The pollen residue was extracted for 6 hours with chloroform in a Soxhlet apparatus. After evaporating the chloroform and drying as above the extract weighed 0.4215 gm. or 8.43 per cent. (*c*) The pollen residue was extracted a second time with chloroform for 6 hours. After evaporating and drying as above the extract weighed 0.0538 gm. or 1.08 per cent. (*d*) The pollen residue was extracted a third time with chloroform for 48 hours. On evaporating and drying as before there was obtained a semicrystalline material which weighed 0.0340 gm. or 0.68 per cent.

The total yield of extract in the above operations was, therefore, 0.8908 gm. or 17.82 per cent. These extracts were united and exhausted with absolute ether. After filtering, evaporating the ether, and drying, the ether-soluble extract weighed 0.6940 gm. or 13.88 per cent.

The ether-insoluble material which remained was a semicrystalline solid which was readily soluble in water. Evidently, therefore, it was neither fat nor lecithin.

Attention is called to the fact that a higher yield of extract was obtained by digesting the pollen in four successive portions of alcohol as shown in Table I than by the above alcohol-chloroform extraction. The ether-soluble part of the alcoholic extract was 14.93 per cent as against 13.88 per cent of alcohol-chloroform extract. The lecithin content of the ether-soluble portion of the alcoholic extract was also higher than in the alcohol-chloroform extract as will be shown below. But only a small amount of the ether-soluble material was phosphatides as shown by the low phosphorus content. The nature of the non-phosphatide part of the ether-soluble portion of the alcoholic extract has not been determined.

It is interesting to note that while the maximum amount of ether extract obtained by direct extraction of the pollen with absolute ether was only 1.58 per cent yet the amount of alcohol-chloroform and the absolute alcohol extracts soluble in absolute ether was from 14 to 15 per cent of the weight of the pollen.

It is probable that the membrane surrounding the pollen grains is nearly impermeable to ether while alcohol and chloroform permeate the membrane and dissolve out fats and phosphatides together with other substances. This assumption would account for the low percentages of ether-soluble material obtained in the direct extraction of the pollen with ether and for the much larger yields of ether-soluble substances in the alcoholic or chloroform extracts.

The difficulty of completely extracting the soluble constituents is greater in the case of pollen than in other plant material because it is practically impossible to rupture the pollen membranes by ordinary trituration. Prolonged grinding in a mortar, even after the pollen has been extracted with ether and alcohol, produces only a small percentage of broken cells.

Phosphatide Content of Corn Pollen.

The absolute ether extract obtained from pollen contained, as previously stated, only a trace of phosphorus. A larger amount of ether-soluble or phosphatide phosphorus was contained in the alcoholic and the alcohol-chloroform extracts.

The phosphorus in the alcoholic extract amounted to 0.19 per cent of the pollen. Nearly all of this phosphorus, or 0.139 per

cent was soluble in ether. Multiplying this number by the usual factor for lecithin we obtain 3.62 per cent of lecithin in pollen. In the alcohol-chloroform extract the ether-soluble phosphorus amounted to 0.104 per cent which corresponds to 2.72 per cent of lecithin. These figures indicate that the phosphatides are more completely removed from pollen by absolute alcohol than by the alcohol-chloroform treatment.

Nitrogen in Corn Pollen and the Nitrogen Distribution in Pollen Extracts.

The total nitrogen in the air-dried pollen was 4.30 per cent.

10 gm. of pollen were extracted with ether in a Soxhlet apparatus for 24 hours. It was then extracted with absolute alcohol for 24 hours. The alcohol was evaporated and the extract was taken up in ether as much as possible, filtered, and the ether evaporated.

The ether extracts were united and were found to contain 0.14 per cent of nitrogen.

The ether-insoluble portion of the alcoholic extract contained 0.18 per cent of nitrogen.

The pollen residue after extracting with ether and alcohol was digested in water, filtered, and washed with water. The water-soluble nitrogen amounted to 0.50 per cent. The pollen residue from the above extractions, after drying contained 3.49 per cent of nitrogen.

The above results are calculated to the original air-dried pollen.

Approximate Composition of Corn Pollen.

The figures given in Table II were obtained on analyzing the pollen obtained from three varieties of corn. The results are calculated to the water-free pollen.

We do not feel that we can give any adequate reason for the striking differences found for starch and sucrose. Several determinations were made in duplicate and triplicate with concordant results. It is not impossible that the difference in composition depends upon varying degrees of ripeness but we tried, as far as possible, to gather all of this pollen when it was just ripe.

It is not improbable that different varieties of corn may produce pollens of different composition. However, until more work

has been done on this subject we would only offer this explanation with some reserve.

TABLE II.

Analysis of Corn Pollen.

Constituent.	Yellow dent corn. Improved Leaming. Pollen gathered 1919.	White flint corn. Luces Favorite. Pollen gathered 1920.	Pop-corn. Pollen gathered 1920.
	per cent	*per cent*	*per cent*
Starch	11.07	19.04	18.03
Nitrogen	4.53	4.43	3.85
Reducing sugar as dextrose	3.50	5.38	4.95
Sucrose	9.09	2.97	14.18
Pentosans	10.60		
Crude fiber	5.35		
Crude fat or ether extract (average)	1.48		
Ash	3.46	3.83	3.13
Phosphorus	0.63		
Sulfur	0.34		
Chlorine	0.19		
Potassium	1.24		

Analysis of the Pollen Ash.

For analysis the pollen was ashed at a low temperature in an electric muffle and a pure white ash was obtained. The result of the analysis is given in Table III.

TABLE III.

Composition of the Pollen Ash from Yellow Dent Improved Leaming Corn.

Constituent.	Per cent.
Phosphorus	18.92
Sulfur	0.69
Chlorine	0.80
Silica, SiO_2	3.76
Calcium	1.02
Magnesium	4.60
Potassium	35.58
Sodium	0.69
Iron	0.25
Aluminum	0.22

Separation of Certain Soluble Constituents of Corn Pollen.

The pollen used in this investigation was obtained from the Improved Leaming variety of yellow dent corn which had been gathered in 1919. It was dried in vacuum over sulfuric acid.

Extraction with Ether.

The pollen, 590 gm. of dry material, was extracted with absolute ether during two 24 hour periods. After evaporating the ether and drying the extract in vacuum over sulfuric acid it weighed 9.0 gm. This is equal to 1.52 per cent of ether extract. The extract was of a dirty green color and of a soft, wax-like consistency. It was not further investigated.

Extraction with Alcohol.

The pollen residue was placed in a 2 liter flask, 1 liter of absolute alcohol was added and the mixture was heated to 60°C. under a reflux condenser for 1 hour. It was allowed to stand at room temperature for 15 hours and then heated to 60° for about 5 hours. It was filtered while hot on a Buchner funnel and washed with absolute alcohol. These operations were repeated three times with fresh portions of alcohol. The pollen residue was reserved for further examination.

Examination of the Alcoholic Solution.

The alcoholic extract was of a greenish yellow color and it measured about 4 liters.

On cooling and standing over night in the ice chest there separated out a small amount of colorless crystals in the bottom of the flask. This material was filtered off and will be referred to later as "Substance A."

The alcoholic solution was concentrated in vacuum at a temperature not exceeding 40° to about 300 cc. During the evaporation of the alcohol a considerable amount of a crystalline substance was deposited in the flask and the quantity increased on cooling and standing over night. The crystals were filtered off and washed in alcohol. This material will be referred to later as "Substance B."

The alcoholic solution was now taken to dryness in vacuum. There remained a thick oily substance which was mixed with some crystalline product. This oily residue was shaken with several portions of absolute ether in which the greater amount of the material dissolved. The ether-insoluble, semicrystalline substance was added to "Substance B" mentioned above.

Preparation of the Amorphous Phosphatide.

The ethereal solution was evaporated to a syrupy consistency and to it were added with constant shaking 600 cc. of acetone. A heavy sticky substance was precipitated which settled to the bottom of the flask. The dark-colored acetone solution was decanted and the residue was washed thoroughly with acetone.

The acetone solution and washings were concentrated to a thin syrup and again poured into 600 cc. of acetone when a further quantity of the sticky substance, similar to the first, separated. After decanting the mother liquor and washing with acetone this precipitate was added to the first amorphous phosphatide.

Preparation of the Crystalline Phosphatide.

The acetone mother liquor was allowed to stand in the ice chest for 2 days. A considerable quantity of nearly colorless, large, thin, plate-shaped crystals separated gradually. The crystals were removed, washed in acetone, and dried in vacuum over sulfuric acid. The dry substance weighed 4 gm. This crystalline material was found to be a phosphatide. It will be described later.

The acetone solution was evaporated to dryness under reduced pressure. There remained a thick oily brown residue which, after drying in vacuum over sulfuric acid, weighed 26 gm. This material still contained a considerable amount of phosphatide because it contained 0.55 per cent of phosphorus and 0.4 per cent of nitrogen, but it was not further examined.

Purification of the Amorphous Phosphatide.

The substance which was precipitated by acetone from the ethereal solution was dissolved in absolute ether. The ether solution was shaken with water and afterwards with a dilute solu-

tion of sodium chloride. The emulsions which formed were broken up with much difficulty by adding sodium sulfate. The solution was finally dried with sodium sulfate, filtered, and the ether evaporated until a thin syrup remained. This was poured with constant stirring into 600 cc. of acetone. The phosphatide separated as a thick, pasty mass. The acetone was decanted and the phosphatide washed several times by thoroughly stirring with acetone. After drying in vacuum over sulfuric acid it weighed 11.5 gm.

The two phosphatide preparations had a combined weight of 15.5 gm. This corresponds to a yield of 2.6 per cent.

The amorphous phosphatide after drying formed a light yellowish brown, hard, brittle mass which could be powdered. It was not very hygroscopic. For analysis it was dried in vacuum over phosphorus pentoxide at the temperature of boiling chloroform. Further drying at 78°C. did not cause any loss in weight. There was no perceptible change in color on drying at the above temperature and the loss in weight was only 1.09 per cent.

Found. P = 3.86, N = 1.53 per cent.
Ratio N : P = 1 : 1.1

The percentage of phosphorus and nitrogen and the N:P ratio corresponded very nearly to the values required for distearyl lecithin.

Hydrolysis of the Amorphous Phosphatide.

Without subjecting the substance to any further purification an attempt was made to determine quantitatively the amounts of choline, glycerophosphoric acid, and fatty acids after hydrolysis. In this experiment we followed the method outlined by Osborne and Wakeman (3) in their study of the hydrolysis of the phosphatide from milk. We used 5.2390 gm. of the dry phosphatide and obtained 0.8519 gm. of choline platinum chloride, 1.2035 gm. of barium glycerophosphate, and 2.2646 gm. of fatty acids. The figures presented in Table IV are calculated from the above values.

The choline platinum chloride after recrystallizing from water contained 32.21 per cent of Pt.

$(C_5H_{14}ONCl)_2PtCl_4$. Calculated. Pt 31.64 per cent.

The barium glycerophosphate was purified by precipitating it from aqueous solution with alcohol until a pure white amorphous preparation was obtained. The air-dried substance lost 8.30 per cent of water on drying at 105°C. in vacuum over phosphorus pentoxide and the weight remained constant on further drying at 130°C. On analysis the dried preparation gave:

Ba = 39.90, P = 9.17 per cent.
$C_3H_7O_6P$ Ba + 2 H_2O. Calculated. Ba = 40.01, P = 9.02 per cent.

The analytical results agree with the theoretical composition of barium glycerophosphate plus 2 H_2O. But the fact that this water could not be driven off at 130°C. in vacuum makes the purity of the preparation somewhat doubtful. Winterstein and Hiestand (4) obtained a barium glycerophosphate of similar composition from the phosphatide which they had isolated from wheat flour. MacLean (5) calls attention to the difficulty of purifying the glycerophosphoric acid prepared from plant phosphatides.

TABLE IV.

Cleavage Products of Amorphous Phosphatide.

Constituent.	Amount found.		Calculated for distearyl lecithin.
	gm.	*per cent*	*per cent*
Choline	0.3335	6.36	14.99
Glycerophosphoric acid	0.6733	12.85	21.31
Fatty acids	2.2646	43.22	70.26

Some evidence was found of the presence of another base besides choline in the phosphatide. After the choline platinum chloride had been filtered off the alcoholic solution was evaporated and the residue was taken up in water. The platinum was precipitated by hydrogen sulfide and the filtrate was evaporated to dryness under reduced pressure. The residue was extracted at room temperature with absolute alcohol which left a small quantity of an insoluble white crystalline substance. This was recrystallized from hot 95 per cent alcohol and was obtained in colorless needle-shaped crystals which weighed 0.13 gm. From this substance a gold double salt was prepared which crystallized from

water in large yellow needles. It contained 49.84 per cent of gold and melted at 132°C. (uncorrected), but we were unable to identify this substance.

On hydrolysis of the phosphatide a mixture of saturated and unsaturated fatty acids was obtained. The percentage of iodine absorbed by the crude fatty acids was 49.01 determined by the Hanus method (6). The fatty acids were saponified and lead soaps were prepared and extracted with ether. The ether-insoluble lead salt was decomposed with hydrochloric acid and extracted with ether. After evaporating the ether, the residue was recrystallized several times from absolute alcohol. The snow-white crystals melted at 63°C. (uncorrected). The melting point of palmitic acid is 62.6°C. and it is probable, therefore, that the saturated acid was nearly pure palmitic acid.

Owing to the small quantity of unsaturated acid its nature could not be determined.

The values found for the cleavage products of the amorphous phosphatide as indicated in Table IV are very much lower than is required for the formula of distearyl lecithin. This might be due to admixed impurities or possibly to the presence of carbohydrates. An attempt was made to determine the amount of carbohydrate present in the phosphatide. After hydrolyzing by boiling with 5 per cent sulfuric acid, as described by Winterstein and Hiestand (7), cooling, and neutralizing with sodium hydroxide only a very slight reduction was obtained on boiling with Fehling's solution. The phosphatide contained, therefore, only a trace of carbohydrate.

A complete analysis of the substance was made when it was found that, in addition to carbon, hydrogen, phosphorus, and nitrogen, it also contained sulfur. The results obtained on analysis are given below.

Found. C 57.78, H 8.53, P 3.86, N 1.53, S 0.68 per cent.

The carbon and hydrogen are much lower than is required for distearyl lecithin and the presence of sulfur would indicate that the substance is a mixture of phosphatide and sulfatide.

Analysis of the Crystalline Phosphatide.

The crystalline phosphatide which separated from the acetone mother liquor was of a slightly yellowish white color. After drying in vacuum over sulfuric acid it was decidedly hygroscopic and on exposure to the air it became sticky. Qualitative analysis showed that it contained phosphorus and nitrogen but no sulfur. On drying at the temperature of boiling chloroform in vacuum over phosphorus pentoxide it lost only 0.93 per cent in weight and the weight remained constant on further drying at 78°C. On analyzing it for phosphorus and nitrogen the following results were obtained.

Found. P 1.74, N 1.53 per cent.
Ratio N:P = 1.95:1.

The N:P ratio is nearly as 2:1 and the substance is, therefore, probably a diaminomonophosphatide.

Lack of time and material has prevented a complete examination of the phosphatides of corn pollen. The results obtained so far indicate that at least two phosphatides are present. We hope to prepare more material and will report on a more complete investigation of the corn pollen phosphatides in a later publication.

Examination of "Substance A."

It was mentioned previously that a small quantity of a colorless crystalline substance separated when the absolute alcoholic extract of pollen was allowed to stand over night. The crystals were filtered off, washed in a little absolute alcohol, and dried in vacuum over sulfuric acid. It weighed 0.2 gm. The substance was insoluble in water and very slightly soluble in cold alcohol, but readily soluble in ether, chloroform, and in hot alcohol.

The substance was twice recrystallized from boiling absolute alcohol from which it separated on cooling in small transparent plates. The dry crystals were snow-white in color and they exhibited a fatty feeling to the touch. It gave in somewhat modified form the Salkowski and Liebermann reaction of cholesterol or phytosterol. The melting point, however, was sharp at 88–89°C. (uncorrected). The small quantity of this substance prevented its identification but the crystal form and melting point correspond to those of myricyl alcohol.

Examination of "Substance B."

This substance separated in crystalline form on concentrating the absolute alcoholic extract of pollen. The crystals were filtered off and washed in alcohol. The substance was readily soluble in water and it crystallized again on adding alcohol to the aqueous solution. Through an accident the larger part of the material was lost, but from the small quantity which was saved we obtained after recrystallizing four times 0.75 gm. of beautiful colorless needle-shaped crystals. It gave the reaction of Scherer and melted at 221°C. (uncorrected), thus showing that the substance was pure inosite.

Quantitative Determination of Inosite in Corn Pollen.

60 gm. of the pollen were digested in 200 cc. of water with frequent shaking for 3 hours. It was then filtered through a layer of paper pulp and washed with water until 450 cc. of filtrate were obtained. The filtrate was yellow in color and it possessed a strong odor of pollen. It was evaporated to 50 cc. on the water bath, filtered, and the inosite was isolated by the method of Mayer (8). The pure colorless characteristic inosite crystals finally obtained weighed 0.5 gm. which corresponds to a yield of 0.83 per cent, but considering the inevitable losses during the isolation and purification it is very probable that corn pollen contains not less than 1 per cent of free inosite. The crystals gave the Scherer reaction and melted at 221°C. (uncorrected). We have no doubt whatever that the substance was pure inosite and the analysis was therefore omitted.

Extraction of the Pollen Residue with 70 Per Cent Alcohol.

The pollen residue which remained after extracting with ether and alcohol, as already described, was digested in 1 liter of 70 per cent alcohol at room temperature, with occasional stirring, for several days. It was then filtered on a Buchner funnel and washed with 70 per cent alcohol until about 1,300 cc. of filtrate were obtained. This solution was concentrated under reduced pressure to about one-half of its volume. It was then heated nearly to boiling and alcohol was added until the solution turned cloudy. After standing in the ice chest several days a considerable amount of a crystalline substance had separated.

The crystals were filtered off and washed in alcohol. They were dissolved in a little water, decolorized with animal charcoal, and again brought to crystallization by adding alcohol. After recrystallizing four times 1.4 gm. of colorless needle-shaped crystals were obtained. The crystal form was characteristic of inosite. The substance gave the reaction of Scherer and melted at 221°C. (uncorrected). Since the reactions and properties of this substance indicated that it was pure inosite the analysis was omitted.

After the above crystals of inosite had separated the mother liquor was concentrated to a thin syrup under reduced pressure at a temperature not exceeding 40°C. The syrup was taken up in a little water and precipitated with a solution of lead acetate. After settling, the precipitate was filtered on a Buchner funnel and washed with water.

The filtrate was freed from lead by hydrogen sulfide and the excess of hydrogen sulfide was removed by a current of air. The solution was then decolorized with animal charcoal and concentrated under reduced pressure. Sulfuric acid was added until the solution contained about 5 per cent of this acid. A concentrated solution of phosphotungstic acid was then added until no further precipitation occurred. After standing for several hours the precipitate was filtered and washed with 5 per cent sulfuric acid.

Unfortunately, the filtrate was lost through an accident which prevented an examination of it for amino-acids and soluble carbohydrates.

The phosphotungstic precipitate was rubbed up in a mortar with an excess of barium hydroxide, filtered, and the precipitate thoroughly washed with water. The filtrate was acidified slightly with sulfuric acid, filtered from barium sulfate, and concentrated under reduced pressure to about 400 cc. The solution was then made up to 500 cc. with water. Nitrogen was determined in this solution by the Kjeldahl method and it was found to contain 0.8470 gm. of nitrogen.

Through fractional precipitation with phosphotungstic acid we were able to separate the nitrogenous constituents into two principal fractions which were identified as choline and *l*-proline. The nitrogen recovered amounted to about 80 per cent, the balance being lost in the processes of separation and purification.

Separation of Choline.

An attempt was made to separate histone bases by the method of Kossel and Kutscher (9) as described by Steudel (10) but only traces of nitrogen were precipitated. After the solution was freed from barium and silver about 4 per cent of sulfuric acid and a very slight excess of phosphotungstic acid were added. The precipitate was filtered after standing over night and washed with 4 per cent sulfuric acid. The filtrate and washings were reserved for the preparation of *l*-proline.

The phosphotungstic precipitate was decomposed with barium hydroxide, the excess of barium removed with carbon dioxide, and the filtrate made up to 500 cc. This solution was found to contain 0.2327 gm. of nitrogen determined by the Kjeldahl method. This would correspond to 2.01 gm. or 0.34 per cent of choline in corn pollen. The solution was concentrated under reduced pressure, filtered from a small quantity of barium carbonate, and evaporated in a vacuum desiccator over sulfuric acid. The thick syrupy residue which remained was taken up in a little alcohol and to it was added an alcoholic solution of picric acid until the solution turned cloudy. On cooling the picrate separated in large needle-shaped crystals. These were filtered off and washed in a little alcohol. The filtrate and washings were concentrated and on cooling a further quantity of crystals were obtained which were added to the first lot. The picrate was recrystallized from a little hot water, filtered, washed with absolute alcohol, and dried in vacuum over sulfuric acid. The dry picrate weighed 2.67 gm. and it melted at 239–240°C. (uncorrected).

The picrate was suspended in about 50 cc. of water and acidified with hydrochloric acid and the picric acid was shaken out with ether. The aqueous solution was concentrated under reduced pressure and finally dried in vacuum over sulfuric acid until a mass of colorless crystals remained. The crystals were very hygroscopic and rapidly liquefied on exposure to the atmosphere. The xanthine and the Weidel reactions and the Kossel reaction for adenine were all negative.

The substance was taken up in about 20 cc. of absolute alcohol, and ether was added gradually until crystallization began. It was then placed in a freezing mixture for 1 hour. The crystals were

filtered, rapidly washed with absolute ether, and dried in vacuum over sulfuric acid. In this manner the substance was obtained in long fine colorless needles. It was very hygroscopic and on exposure to the air the crystals rapidly liquefied. The crystals did not melt or show any change when heated to 260°C.

The alcoholic solution of the substance gave an orange-colored precipitate with platinic chloride. The platinum double salt was prepared and after recrystallizing from hot water to which about 15 per cent of alcohol was added it was obtained in orange-colored octahedral crystals. The air-dried double salt melted with decomposition at 232°C. (uncorrected).

On analysis, after drying at 105°C. in vacuum over phosphorus pentoxide, values were obtained which agree with the theoretical composition of choline platinum chloride.

0.1742 gm. substance: 0.0548 gm. Pt.

$(C_5H_{14}NO\ Cl)_2\ Pt\ Cl_4$.	Calculated.	Pt 31.64 per cent.
	Found.	" 31.45 " "

Preparation of l-Proline.

After the choline fraction had been precipitated by phosphotungstic acid, the filtrate was freed from sulfuric and phosphotungstic acids by barium hydroxide. The filtered solution was evaporated under reduced pressure and the residue was taken up in a little hot water. A small quantity of barium carbonate was filtered off and the filtrate made up to 100 cc. with water. Nitrogen was determined by the Kjeldahl method and the solution was found to contain 0.4396 gm. of nitrogen.

The solution was acidified with hydrochloric acid and concentrated under reduced pressure and finally dried in vacuum over sulfuric acid. A thick syrup remained which on scratching with a glass rod immediately crystallized. The crystals were digested in a little absolute alcohol, filtered on a Buchner funnel, and washed with a little cold absolute alcohol and ether. After drying in the air the substance weighed 3 gm. and it was a nearly pure white crystalline powder. It was very soluble in water and in hot 95 per cent alcohol. It also dissolved readily in hot absolute alcohol and on cooling it separated in colorless prisms. The alcoholic solution gave no precipitate with platinic chloride or with picric acid. The substance showed an acid reaction on litmus and on ignition it left no residue.

The substance was twice recrystallized from hot absolute alcohol and was obtained in beautiful colorless prisms. When heated in a capillary tube it melted with gas formation at 206–207° (uncorrected). It contained nitrogen, but sulfur, phosphorus, and halogens were absent. Boiled with copper oxide it gave off a peculiar odor and a deep blue-colored solution resulted. This solution was filtered and evaporated when a deep blue-colored amorphous copper salt was obtained which was completely soluble in alcohol.

The substance was analyzed after drying at 105°C. in vacuum over phosphorus pentoxide. There was no loss in weight on drying.

0.1470 gm. substance: 0.1051 gm. H_2O and 0.2791 gm. CO_2.
0.1560 " " 16.9 cc. of nitrogen at 16° and 731 mm.
Found. C 51.78, H 8.00, N 12.30 per cent.
For $C_5H_9NO_2$ = 115.
Calculated. C 52.17, H 7.82, N 12.17 per cent.

In aqueous solution the substance had a specific rotation of −69.69°.

The properties and composition of this substance agree with those of *l*-proline although the rotation is lower than given by Fischer (11). The identity with proline was established by converting it into *dl*-proline as described by Fischer (11). The copper salt was obtained in the form of the characteristic deep blue-colored crystals.

The following results were obtained on analysis after drying at 105° in vacuum over phosphorus pentoxide.

For $(C_5H_8NO_2)_2Cu + 2H_2O$ = 327.5. Calculated. H_2O 10.99 per cent.
Found. " 10.84 " "
For $(C_5H_8NO_2)_2Cu$ = 291.5. Calculated. Cu 21.78 per cent.
Found. " 21.73 " "

Some of the *l*-proline was treated with phenylisocyanate in alkaline solution and the resulting compound was converted into the anhydride or hydantoin by heating with 4 per cent hydrochloric acid. After recrystallizing four times from aqueous alcohol, the reaction product was obtained in the form of fine colorless needles which melted at 143°C. (uncorrected). Proline hydantoin melts at 143°C.

Corn pollen contains a relatively large amount of proline. The solution from which the proline was prepared contained 0.4396 gm. of nitrogen which corresponds to 3.6 gm. or 0.6 per cent of free *l*-proline in the pollen.

Phosphorus in the Extracted Corn Pollen.

The pollen residue after extracting with ether, absolute alcohol, and 70 per cent alcohol was analyzed for phosphorus. Total phosphorus was determined after destroying the organic matter by the Neumann method. The total soluble and the inorganic phosphorus were determined in the extracts obtained on digesting the pollen residue during 5 hours in 1 per cent hydrochloric acid. The value for the organic phosphorus soluble in 1 per cent hydrochloric acid was obtained by subtracting the inorganic from the total soluble phosphorus. The results are given in Table V.

TABLE V.

Forms of Phosphorus in Corn Pollen after Extracting It with Ether, Absolute Alcohol, and 70 Per Cent Alcohol.

Total phosphorus.	Total soluble phosphorus.	Inorganic phosphorus.	Organic phosphorus.
per cent	*per cent*	*per cent*	*per cent*
0.43	0.29	0.22	0.07

The organic phosphorus in Table V corresponds to the phytin phosphorus as determined in other plant material. Judging by the very small difference between the total soluble and the inorganic phosphorus in pollen it appears rather doubtful if phytin or inosite hexaphosphoric acid is present in this material.

Nitrogen in the Extracted Corn Pollen.

The pollen residue remaining after extracting with ether, absolute alcohol, and 70 per cent alcohol was analyzed for nitrogen. The material was extracted with the solvents mentioned in the table and the results obtained are given briefly in Table VI.

In earlier investigations the presence of malic acid has been reported in the pollen of *Phœnix dactylifera* by Fourcroy (12) and in the pollen of *Typha latifolia* by Braconnot (13) and Kresling (14) found tartaric and malic acid in the pollen of *Pinus sylvestris*.

In the case of corn pollen we were unable to obtain any evidence of the presence of any of these acids. The only acid which we could find in aqueous extracts of corn pollen was phosphoric acid in the form of calcium phosphate.

The soluble constituents which were isolated from corn pollen and identified are given in Table VII. The percentages are calculated to the dry pollen.

TABLE VI.

Nitrogen in the Extracted Corn Pollen.

Total nitrogen.	Nitrogen soluble in 1 per cent NaOH. Digested on water bath for 24 hours.	Nitrogen soluble in 1 per cent hydrochloric acid. Digested on water bath for 24 hours.	Nitrogen soluble in 5 per cent NaCl. Digested at 25°C. for 24 hours.	Nitrogen soluble in distilled water. Digested at 25°C. for 24 hours.
per cent	*per cent*	*per cent*	*per cent*	*per cent*
5.40	4.93	3.20	0.40	0.20

TABLE VII.

Soluble Identified Constituents Occurring in Corn Pollen.

Substance.	Per cent.
Amorphous phosphatide	1.94
Crystalline phosphatide	0.67
Inosite	0.83
Choline	0.34
l-Proline	0.60
Myricyl alcohol	Trace.

Some attempts were made towards the isolation of proteins, nucleic acid, and certain carbohydrates from pollen but these experiments are still incomplete. We hope to present a fuller report on the above constituents in a later publication.

SUMMARY.

The approximate composition of the pollen from three varieties of corn has been determined and the results indicate a difference in the composition of the pollen from different varieties of corn. A complete analysis of the ash of the pollen from one variety of corn is given.

Evidence is presented which indicates the presence of at least two phosphatides in corn pollen. One was an amorphous substance which also contained sulfur but the other was a crystalline phosphatide.

Relatively large quantities of free inosite, *l*-proline, and choline occur in corn pollen.

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THE RÔLE OF CEPHALIN IN BLOOD COAGULATION.

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The process of blood clotting is very complex. As it occurs normally, it is the result of many factors not well understood. The progress made in recent years was due to the fact that ways were found to separate the many factors into groups, some of which control definite phases of the complex process of blood clotting. The phase best understood is that of the conversion of fibrinogen into fibrin. Four substances take part in this reaction. One is the substrate, and the other three combine to bring about the transformation of the substrate from a soluble into an insoluble state. Alexander Schmidt understood the process correctly. More recent workers brought out many of the details of the process. The most recent contributions were made by Bordet and his collaborators, and by Howell and his coworkers.

The terms applied to each of the factors differed with the individual author. Since the blood clotting experiments here reported were carried out by a worker of Bordet's school, the nomenclature employed in this publication is of that school. In terms of that school the process of fibrin formation is expressed by the following diagram.

$$\left.\begin{array}{l}\text{Plasma at the}\\ \text{moment of coagulation}\\ \qquad\text{or}\\ \text{serum after coagulation}\end{array}\right\}\text{serozyme}$$

$$\text{in presence of Ca ions} \longrightarrow \text{thrombin}$$

$$\left.\begin{array}{l}\text{Platelets}\\ \qquad\text{or}\\ \text{tissue extracts}\\ \qquad\text{or}\\ \text{certain lipoids}\end{array}\right\}\text{cytozyme}$$

$$\text{thrombin} + \text{Fibrinogen} = \text{fibrin}$$

Howell accepts the interplay of all four of the substances in the process of blood clotting but holds a different view on the rôle of cytozyme, which, according to Howell, plays no part in the actual transformation of fibrinogen into fibrin. Howell also disagrees with other workers in his view on the chemical nature of cytozyme. Alexander Schmidt, Wooldridge, Bordet, Delange and others regarded the substance as lecithin. This opinion was based on the thermostability of the substance and on its solubility in alcohol. Howell on the contrary came to the conclusion that the active substance was another phosphatide; namely, cephalin. Howell and his coworkers have also made an attempt to associate the activity of the phosphatide with a definite peculiarity of its chemical structure. At the time of the work of Howell it was generally accepted that the unsaturated acid entering into the structure of cephalin differed from that of lecithin. From cephalin, linolic acid was isolated and from lecithin, oleic acid. According to Howell and McLean the higher unsaturation of the fatty acid is the factor which lends to cephalin its property of being an agent in the formation of thrombin.

The present communication is a mere note dealing not with the entire problem of fibrin formation but only with the chemical nature of cytozyme. Is cytozyme lecithin or cephalin? Since the work of Howell and his coworkers, the knowledge of the chemical structure of phosphatides has made considerable progress. In the light of this progress the conclusions regarding the chemical nature of cytozyme required reinvestigation.

The recent work on lecithin has brought out the fact that there exist forms of this substance which contain in their molecule a fatty acid of still higher unsaturation than the one previously isolated from cephalin. In the light of the theory of Howell and McLean, one might have expected the new form of lecithin to play the same part as cephalin in fibrin formation.

Furthermore, recent work on cephalin has brought out the fact that the material handled by the older writers under the name of lecithin was in reality a complex mixture and not a uniform substance. The components of this mixture were found to be identical in character with those of another complex mixture described by previous writers under the name of cuorin, or heparphosphatide. Cuorin and the cephalin of the older writers

differed one from another in the proportions of some of their components. Both substances were found to consist of true cephalin, true lecithin, and of the same substances in a state of partial decomposition. The character of the decomposition products varied from sample to sample. Yet, Howell and his coworkers observed that cephalin and cuorin acted in the process of blood coagulation antagonistically to one another.

On the other hand, a substance was recently prepared which was free from the decomposition product of lecithin and of cephalin and which contained 75 per cent of undecomposed cephalin and 25 per cent of undecomposed lecithin. Whether or not the substance contained impurities undetectable by the present methods of analysis, cannot be stated.

It is self-evident that it became important to compare the cytozymic function of the three substances; namely, of ordinary lecithin, of lecithin which contained the fatty acid of a high degree of unsaturation, and of the new cephalin material. In a way also the present materials were mixtures. Ordinary lecithin contains a small proportion of the new form. The new form still contained a very small proportion of the older type. The cephalin contained a small proportion of lecithin. Yet even such material was sufficient to bring out the fact that lecithin, regardless of its form and of its origin, possesses no cytozymic action. On the other hand, material containing 75 per cent of undecomposed cephalin and 25 per cent of lecithin possesses unusually high cytozymic action. It is still active in a concentration of 5 (10^{-7}).

The coagulation experiments were carried out by Dr. Gratia who followed the routine customary in Bordet's school. The plan and the details of the experiments follow.

EXPERIMENTAL PART.

Oxalated plasma from which most of the platelets have been removed by centrifugation contains only a small amount of cytozyme and consequently clots very slowly when recalcified, but clots quickly if some cytozyme is given back in form either of platelet suspension, tissue juice, or lipoidic tissue extract. This offers means of testing the cytozymic properties of a given lipoid by measuring the accelerating influence of the lipoid on the coagulation of a plasma almost free from platelets.

When an oxalated plasma has been strongly centrifugalized and then recalcified, the few remaining platelets contain just enough cytozyme to react with but a small part of the serozyme and thus yield only a small amount of thrombin. The plasma clots slowly and a great excess of unutilized serozyme is found in the serum after coagulation. Such a serum is rich in serozyme and is an excellent reagent to test the cytozymic properties of a given lipoid. If cytozyme even in very small amount is added to this serum, an active production of thrombin immediately results and this mixture is able to clot an equal volume of fibrinogen or oxalated plasma in a few minutes. This is the serozyme-cytozyme reaction of Bordet and Delange.

In our researches we have submitted our different lipoids to both tests. The materials used were prepared as follows:

Preparation of the Reagents.

1. Lipoidic Emulsions.—1 per cent emulsions of our three lipoids were made in saline solution. As a control a similar 1 per cent emulsion was made with lipoidic extract of tissue which was known to possess strong cytozymic properties. When necessary, higher dilutions of these suspensions were made in the course of the experiments.

2. Oxalated Plasma Free from Platelets.—A rabbit was carefully bled from the carotids with a paraffined cannula. Precautions were taken to avoid the contact of the blood with tissue juice and 9 parts of blood were received in 1 part of a 1 per cent solution of sodium oxalate in saline solution, and thoroughly mixed. This 1 per cent oxalated blood was centrifugalized at high speed during 1 hour and the clear supernatant plasma removed from the cells with a pipette. For use in the experiments 1 part of this oxalated plasma (O. P.) was recalcified with 4 parts of a 0.35 per cent solution of calcium chloride in saline solution (Ca).

3. Serum Rich in Serozyme.—A few cc. of oxalated plasma were recalcified as above described. When coagulation began, the recalcified plasma was defribinated with a glass rod. The serum obtained was kept at room temperature until the next day. As thrombin is very labile, the small amount of thrombin left after this very slow coagulation disappears quickly and the next day the serum containing nothing but a large excess of serozyme is ready for use.

4. Fibrinogen.—Instead of the so called pure solution of fibrinogen, "dioxalated plasma" (F) was used as a test for thrombin. This very convenient reagent was prepared according to the technique of Bordet and Delange; *i.e.*, 1 part of 1 per cent oxalated plasma was diluted with 4 parts of a 2 per cent solution of sodium oxalate in saline solution.

A. Egg Lecithin.

Experiment I.

0.25 cc. O.P.	+ 1 drop	saline solution	+ 7 cc.	Ca	=	110′
0.25 " "	+ 1 "	egg lecithin	+ 7 "	"	=	90′
0.25 " "	+ 1 "	cytozyme	+ 7 "	"	=	20′

Egg lecithin exerts only a slight accelerating influence on the coagulation of recalcified oxalated plasma.

Experiment II.

0.25 cc. serozyme	+ 1 drop	saline solution	..5′...	+ 0.25 cc. F	= ∞
0.25 " "	+ 1 "	egg lecithin	..5′...	+ 0.25 " "	= still fluid after 5 hrs.; soft clot after 24 hrs.
0.25 " "	+ 1 "	cytozyme	...5′...	+ 0.25 cc. F	= 2′

Whereas after 5′ a mixture of serum rich in serozyme together with cytozyme contains a sufficient quantity of thrombin to clot an equal volume of oxalated plasma in 2′, a similar mixture of serozyme with lecithin contains only a practically negligible amount of thrombin that yields hardly a soft clot after 24 hours.

The 1 per cent emulsion of lecithin is rather viscous. Dilutions of the lecithin as well as of the cytozyme emulsions were made, 1/10, 1/100, 1/1,000, and compared.

Experiment III.

0.25 cc. serozyme	+ 1 drop	cytozyme 1/10	...5′...	+ 0.25 cc. F	= 2′
0.25 " "	+ 1 "	lecithin 1/10	...5′...	+ 0.25 " "	= ∞
0.25 " "	+ 1 "	cytozyme 1/100	...5′...	+ 0.25 " "	= 8′
0.25 " "	+ 1 "	lecithin 1/100	..5′...	+ 0.25 " "	= ∞
0.25 " "	+ 1 "	cytozyme 1/1,000	..5′...	+ 0.25 " "	= 25′
0.25 " "	+ 1 "	lecithin 1/1,000	..5′...	+ 0.25 " "	= ∞

Egg lecithin is thus inactive at higher dilutions. In the following series the tests were allowed to react at longer intervals.

Experiment IV.

0.25 cc.	serozyme	+ 1 drop	lecithin	... 5′...	+ 0.25 cc.	F	=	∞
0.25 "	"	+ 1 "	"	...10′...	+ 0.25 "	"	=	∞
0.25 "	"	+ 1 "	"	...15′...	+ 0.25 "	"	=	∞
0.25 "	"	+ 1 "	"	...20′...	+ 0.25 "	"	=	∞
0.25 "	"	+ 1 "	"	...45′...	+ 0.25 "	"	=	∞

The results again were negative. The following series aims to establish whether lecithin in any way affected the potency of the cytozyme.

Experiment V.

0.25 cc. serozyme + 1 drop cytozyme + 1 drop saline solution ..5′.. + 0.25 cc. F = 2′

0.25 cc. serozyme + 1 drop cytozyme + 1 drop lecithin ....5′.... + 0.25 cc. F = 2′

0.25 cc. serozyme + 1 drop cytozyme + 3 drops lecithin ....5′.... + 0.25 cc. F = 2′

0.25 cc. serozyme + 1 drop cytozyme 1/10 + 1 drop saline solution ..5′.. + 0.25 cc. F = 2′

0.25 cc. serozyme + 1 drop cytozyme 1/10 + 1 drop lecithin ..5′.. + 0.25 cc. F = 2′

0.25 cc. serozyme + 1 drop cytozyme 1/100 + 1 drop saline solution ..5′.. + 0.25 cc. F = 10′

0.25 cc. serozyme + 1 drop cytozyme 1/100 + 1 drop lecithin ..5′.. + 0.25 cc. F = 10′

The results show that there was no appreciable accelerating inhibiting influence of egg lecithin on the action of cytozyme.

Conclusion.—Egg lecithin has practically no cytozymic properties. The extremely small action observed in Experiments I and II must very likely be due to traces of the active substance still present as an impurity in the egg lecithin material.

B. Liver Lecithin.

Identical experiments were repeated with the liver lecithin with similar results. Thus the liver lecithin is not more active than egg lecithin.

C. Mixtures of Pure Cephalin and Pure Lecithin.

Experiment VI.

0.25 cc.	O.P.	+ 1 drop	saline solution	+ 1 cc.	Ca	= 50′
0.25 "	"	+ 1 "	cytozyme	+ 1 "	"	= 11′
0.25 "	"	+ 1 "	cephalin	+ 1 "	"	= 10′

The mixture containing 65 per cent of pure cephalin has a marked accelerating effect on the coagulation of recalcified oxalated plasma.

Experiment VII.

0.25 cc.	serozyme	+ 1 drop	saline solution		...5'...	+ 0.25 cc. F	= ∞
0.25 "	"	+ 1 "	cytozyme		...5'...	+ 0 25 " "	= 2'
0.25 "	"	+ 1 "	cephalin		...5'...	+ 0.25 " "	= 1'
0.25 "	"	+ 1 "	"	1/10	...5'...	+ 0 25 " "	= 1'
0.25 "	"	+ 1 "	"	1/100	...5'...	+ 0.25 " "	= 3'
0.25 "	"	+ 1 "	"	1/1,000	...5'...	+ 0.25 " "	= 25'
0.25 "	"	+ 1 "	"	1/10,000	...5'...	+ 0.25 " "	= ∞

1 drop of 7 per cent cephalin emulsion, even diluted 1:1,000, is still able to give a positive result. The calculated amount of material contained in this drop is about 1/20,000 of a mg. This will give an idea of the extraordinary cytozymic activity of the mixture of cephalin and lecithin.

THE HEAT OF REACTION OF OXYGEN WITH HEMOGLOBIN.

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A quantitative understanding of the physical chemistry of oxyhemoglobin requires a knowledge of its heat of formation from oxygen and hemoglobin. With the help of this quantity there may be deduced certain thermodynamic properties of the compound, provided further complexities do not mask the application of simple principles. The present paper gives the results of new measurements of the heat developed when oxygen combines with dissolved hemoglobin, and serves to demonstrate the presence of such complexities. The method used in the thermochemical study of gas-liquid reactions is described, and values obtained by it for the following other reactions are included in the data:

Carbon dioxide + water.
" " + solutions of alkalies.
Oxygen + pyrogallol solutions.
Carbon monoxide + hemoglobin solutions.
" dioxide + blood.

Apparatus.

Gas at a standard temperature and pressure was bubbled through the reacting solution contained in an insulated calorimeter immersed in a thermostat. Temperature changes were read upon a Beckmann's thermometer.

The apparatus is represented in Fig. 1. A silvered Dewar flask of about 230 cc. capacity served as the calorimeter ves-

* The data contained in this paper are taken from a thesis presented by Edward F. Adolph to the faculty of Harvard University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

sel. It was 3 cm. in diameter and 30 cm. deep, so that a long exposure was given to gas bubbling upward. It was closed by a rubber stopper, through which passed the thermometer, an inlet tube reaching to the bottom and ending in a finely perforated bulb, and an outlet tube opening to the air.

The Beckmann's thermometer was graduated in hundredths of a degree Centigrade, and was read to 0.001°. It was calibrated for the range 20.5 to 22.5°C. in an adiabatic calorimeter by comparison with a platinum resistance thermometer in the laboratory of Professor T. W. Richards. We are indebted to Mr. Bridgeman for aid in this calibration. At nine points of comparison the largest correction found for 0.25° was $\pm 0.003°$, and over a range of 2.0° the correction was $+0.0056°$.

The thermostat held 50 liters of water which was maintained at 22°C. $\pm$ 0.02°. A toluene regulator with a mercury-platinum contact was connected to a relay and under its control a set of lamps supplied heat intermittently. A separate current ran a motor which turned a stirrer.

The gases used were oxygen, hydrogen, carbon dioxide, and carbon monoxide. The oxygen supply was drawn from a pressure tank, and contained no carbon dioxide or carbon monoxide. Before use it passed through two wash bottles which contained sodium hydroxide solution. Carbon dioxide also was obtained from a tank. It contained less than 0.5 per cent of air, and was washed only in water. Hydrogen was generated in a small Kipp generator from zinc and hydrochloric acid. After the generator had started, there was no access for air until the zinc was replenished. The gas passed through two wash bottles containing strong solutions of pyrogallol and sodium hydroxide. Carbon monoxide was generated by dropping formic acid into sulfuric acid. It was washed in three bottles of pyrogallol and sodium hydroxide solution.

With each gas a reservoir bottle of 10 liters capacity was filled. A constant flow of gas from the reservoir toward the calorimeter was secured by running water into the reservoir from a second bottle raised above it. Into the second bottle water was run from a faucet at such a rate that the levels in both bottles rose equally, and thus the hydrostatic pressure was constant throughout the flow of gas. To refill the reservoir the water was poured

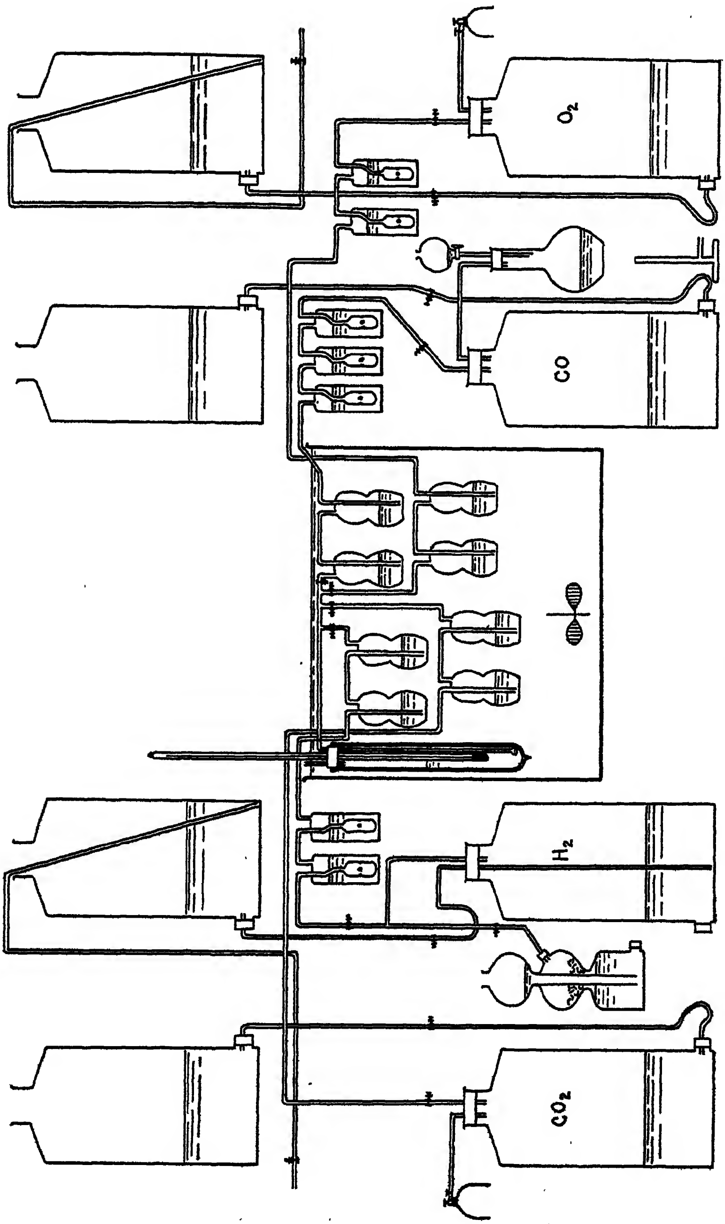

FIG. 1. Diagram of thermochemical apparatus.

from the upper bottle; and this bottle was lowered to receive the water displaced by gas entering the reservoir.

During an experiment the gas left its reservoir at a rate of 400 cc. per minute. It passed through its special wash bottles, and then through a pair of wash bottles immersed in the thermostat. Each gas had a separate pair of these, all containing 0.9 per cent NaCl solution. In them the gas attained the temperature of the bath (22°C.), and was completely saturated with moisture at the vapor tension of this solution. The four gas systems united at the entrance to the calorimeter, and by the manipulation of the necessary stop-cocks one gas flow could be substituted for another in about 30 seconds. The rapid bubbling of gas served to stir the reacting solution in the Dewar flask.

The tube leading out from the calorimeter maintained atmospheric pressure within, and served for sampling the solution by the mere insertion of a pipette.

The foaming of solutions such as hemoglobin and blood was sufficiently inhibited by the addition of a few drops of octyl alcohol.

EXPERIMENTAL PROCEDURE.

A known amount of solution (usually 100 cc.), at about 22°C., was run into the calorimeter. A non-reacting gas was made to flow through it, and thermometer readings were recorded every 60 seconds. The non-reacting gas exhibited the rate of temperature change due to all constant influences, of which the chief was the small difference in temperature between calorimeter and thermostat. This was the fore period, usually lasting 5 minutes. Then the reacting gas was run in and the reaction carried to completion, which required 8 to 12 minutes in most instances. A final period was obtained by continuing the passage of the reactive gas after all chemical change had occurred.

At completion of an experiment, the thermometer readings were plotted against time. A line through the readings of the final period was extrapolated back to the time when the reaction began (Fig. 4). The temperature difference between the extrapolated and the initial readings represents approximately that due to chemical action. For inorganic reactions a greater accuracy was gained by taking the cooling curve for the first half of the reaction period from the readings of the fore period, and only

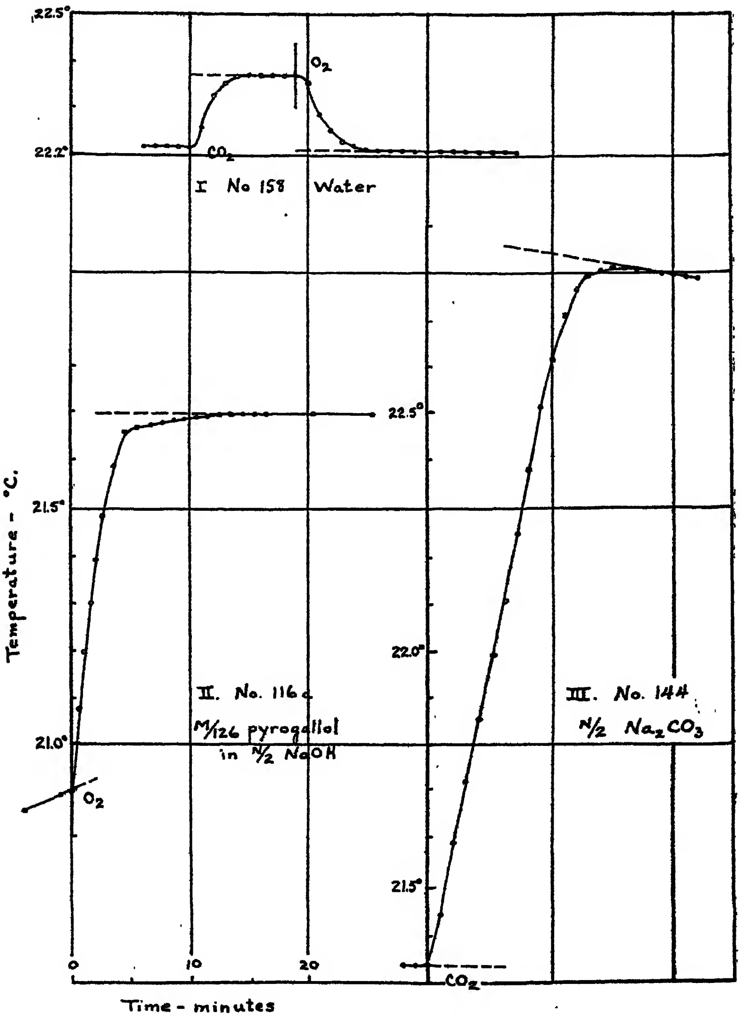

FIG. 2. Heats evolved in dissolving a gram molecule of gas in water at various temperatures; calculated from the solubility data.

the last half from those of the final period (Fig. 2). For rises of temperature of more than 0.5° the method of Pfaundler (1) was used to calculate the cooling correction.

Before calculating the heat production it was necessary to know the heat capacities. The capacity of the calorimeter was roughly determined by three methods: causing solutions of hydrochloric acid and of sodium hydroxide to react in it; introducing water at known temperatures; and calculating the heat capacity of the glass of which it was made. When containing 100 cc. of solution with the thermometer and inlet tube in place, the heat capacity of the calorimeter was 14 calories per degree Centigrade.

The heat capacity of the solutions was calculated. For inorganic solutions the available data (2) were plotted, and the capacity corresponding to the concentrations read off from the curve. For purified hemoglobin solutions a calculation was made on the assumption that the protein has a specific heat of 0.4 calorie per degree Centigrade per gram when dried. For defibrinated blood, concentrated corpuscles, and serum, the values of Hillersohn (3) and of Bordier (4) were used.

Experiments with Inorganic Solutions.

Heats of Solution of Gases.—The rise of temperature when carbon dioxide passes into water, and its fall when oxygen displaces the carbon dioxide from saturated solution, was measured. Assuming that the solubility of carbon dioxide at 22°C. and 760 mm. pressure is 83 volumes per cent (5) the heat of solution was calculated (Table I). The average result of eleven determinations at 22°C. is +4,690 calories per gram molecule of carbon dioxide (standard deviation 2.9 per cent). The chief error in these determinations is the assumed solubility for carbon dioxide. Thomsen (6) found +5,882 calories developed at 19°C., Berthelot (7) found + 5,600 calories at 15°C.

Owing to the small solubility in water of oxygen, nitrogen, hydrogen, and carbon monoxide, it was not possible to measure their heats of solution. The "isochore" of van't Hoff (8) furnishes a method of calculating them, however, for the variation of their solubility with temperature is known from the data of Bohr and Bock (5) and of Winkler (9). Van't Hoff showed that if K_1 and K_2 are the equilibrium (solubility) constants at two absolute temperatures T_1 and T_2, R is the gas constant in heat

TABLE I.

Heat of Solution of CO_2 in Water.

(1) No.	(2) Corrected rise in temperature.	(3) Heat developed per gram molecule CO_2.	(4) Average heat of solution per gram molecule CO_2.
	°C.	*calories*	*calories*
157	0.163	4,940	
158	0.153	4,630	
196	0.154	4,670	
199	0.153	4,630	
222	0.150	4,540	
260	0.150	4,540	+4,690
260a	0.153	4,630	
147	−0.161	4,880	
157a	−0.158	4,780	
158a	−0.160	4,840	
199a	−0.150	4,540	

Formula for calculation: $(3) = \dfrac{(2) \times 112 \times 22,400}{0.829 \times 100}$

units, and Q is the total energy change expressed as heat evolved, then

$$Q = \frac{R\,T_1T_2}{T_1 - T_2} \times \log_e \frac{K_1}{K_2}$$

Values of Q, calculated for 5° intervals of temperature, are plotted in Fig. 3 for each of five gases. Reading from these curves, the values at 22°C. turn out to be:

O_2 +3,100 calories per gram molecule.
N_2 +3,000 " " " "
H_2 +1,200 " " " "
CO +2,800 " " " "
CO_2+5,000 " " " "

The calculated value for carbon dioxide agrees well with the experimental value. None of the gases is appreciably ionized in solution.

Heats of Reaction of CO_2 with Alkalies in Solution.—In these reactions the temperature rise could be made sufficient to yield very accurate results. These are given in Table II. It will be seen that the average value for the reaction of the dissolved gas

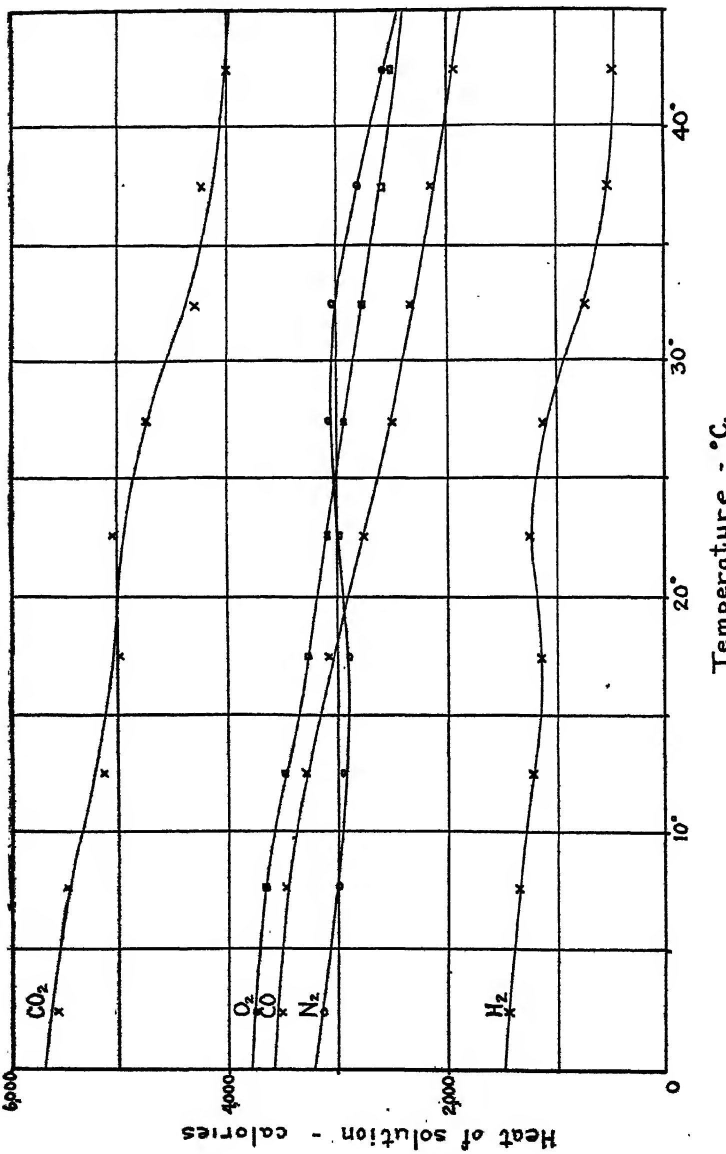

FIG. 3. Temperature changes in the course of three thermochemical measurements.

with sodium hydroxide or potassium hydroxide solutions is +10, 690 calories per gram molecule at 22°C. The standard deviation among ten determinations is 1.0 per cent. Thomsen (6) obtained the value +11,016 calories at 18°C., and Berthelot (7)

TABLE II.

Heat of Reaction of CO_2 with Alkalies.

(1) No.	(2) Solution.	(3) Solution.	(4) Temperature rise corrected for cooling.	(5) Heat capacity per degree.	(6) Heat due to CO_2 dissolved in the solution.	(7) Heat developed per gram molecule of combined CO_2.	(8) Average heat of reaction.
		cc.	*°C.*	*calories*	*calories*	*calories*	*calories*
119	0.298 M NaOH.......	50	4.282	56	8.3	15,480	+10,600
120	0.298 M "	50	4.209	56	8.3	15,220	
121	0.298 M "	50	4.166	56	8.3	15,060	
122	0.298 M "	50	4.183	56	8.3	15,110	
123	0.298 M "	50	4.211	56	8.3	15,240	
124	0.298 M "	50	4.214	56	8.3	15,250	
132	0.149 M "	100	2.216	111	16.8	15,270	
136	0.149 M "	100	2.225	111	16.8	15,350	
138	0.181 M KOH........	100	2.706	111	16.8	15,600	
139	0.181 M "	100	2.665	111	16.8	15,350	
142	0.250 M Na_2CO_3.....	100	1.513	109	16.2	5,950	+1,250
143	0.250 M "	100	1.541	109	16.2	6,070	
144	0.250 M "	100	1.481	109	16.2	5,810	
243	0.067 M Na_2HPO_4....	100	0.323	111	17.0	2,830	−1,900
257	0.067 M "	100	0.298	111	17.0	2,420	
261	0.067 M "	100	0.328	111	17.0	2,910	
258	0.067 M KH_2PO_4.....	100	0.147	111	17.0	0	0

Formula for calculation: $(7) = \dfrac{\{[(4) \times (5)] - (6)\} \times 1,000}{(2) \times (3)}$

+11,100 calories at 18°C., for the reaction of solutions of carbonic acid with solutions of sodium hydroxide.

The heat of neutralization of strong acids and strong bases at 22°C. is +13,600 calories. Using the above experimental results, the heat of ionization of carbonic acid to bicarbonate is

−2,910 calories. Thomsen (6) found it to be −2,800 calories at 18°C., and Kendall (10) calculated by the isochore that it would be −2,830 calories at 22°C.

Heat of Oxidation of Pyrogallol Solutions.—The chemistry of the reaction of oxygen with pyrogallol is not known, and varies with dilution and other factors. Berthelot (11), however, has shown that each molecule of sodium pyrogallate absorbs 3 atoms of oxygen under most conditions. No inactive gas was used in the fore period, but oxygen was bubbled through a solution of 0.5 M sodium hydroxide, and after 5 minutes a weighed amount of crystalline pyrogallol was added (Table III).

TABLE III.

Heat of Oxidation of Pyrogallol.

(1) No.	(2) Pyrogallol.	(3) Solution.	(4) Temperature rise.	(5) Heat capacity.	(6) Heat developed per gram molecule pyrogallol.	(7) Average heat of reaction.
	gm.	*cc.*	*°C.*	*calories/degree*	*calories*	*calories*
114	0.050	100	0.288	112	79,500	+65,000
114a	0.100	100	0.825	112	113,700	
115	0.050	50	0.712	57	99,800	
116	0.050	50	0.810	57	113,500	+95,900
116a	0.050	50	0.783	57	109,700	
117	0.050	50	0.824	57	115,300	

$$\text{Formula for calculation:}\quad (6) = \frac{(4) \times (5) \times 126}{(2)}$$

The heat of solution of pyrogallol, and its heat of complete neutralization, were measured by Berthelot (12) and by de Forcrand (13). Averages of their values (−3,590 and +13,440) were subtracted and a further correction was made for the heat of solution of 3 gram atoms of oxygen, to give the final result of +95,900 calories.

The three varieties of gas reactions studied above indicate the reliability of the method, and demonstrate that it can attain an accuracy of about 1 per cent. Such an accuracy was deemed more than sufficient for the study of the oxygenation of hemoglobin in solution.

Experiments with Hemoglobin Solutions and Blood.

Heat of Reaction of Oxygen and Carbon Monoxide with Hemoglobin Solutions.—The quantitative results depend upon the fact that 1 molecule of oxygen or of carbon monoxide combines chemically with that amount of hemoglobin which contains 1 atom of iron. The molecular weight of hemoglobin has been assumed to be 16,700. Corrections have been made for the heats of solution of the gases, both in measuring heats of reaction and in comparing the values of the equilibrium constant for oxyhemoglobin (K) and for carbon monoxide hemoglobin (k) at various temperatures.

Several investigators have measured the heats of reaction of oxygen and of carbon monoxide with hemoglobin in blood. Without attempting a critique of their methods, a summary of their results is presented in Table IV. In addition to the experiments of four authors included in the table, the work of Camis (18) and of Meyerhof (19) should be mentioned. Camis obtained a negative heat of formation of oxyhemoglobin, though his results have usually been interpreted otherwise. Meyerhof found roughly that when fresh blood was oxygenated, the heat produced just balanced that absorbed due to carbon dioxide carried out of solution.

To avoid as many complications as possible, it was decided to work chiefly with purified hemoglobin solutions. Washed corpuscles from defibrinated beef blood were dialyzed by a method which has been already described (20). Before use the solution of dialyzed hemoglobin was boiled at 40°C. *in vacuo* to remove all gases. It was cooled to 22°C., measured into the calorimeter without exposure to air, and subjected to a stream of hydrogen. After a few minutes the flow of gas was stopped while a sample of solution for analysis was pipetted from the calorimeter. At the end of the passage of the reacting gas a second sample was taken for analysis.

All oxygen and carbon monoxide analyses were performed by the method of Van Slyke (21). In every case a correction was made for dissolved gases. There is some evidence that the quantities thus measured may be too large (22). Carbon dioxide was measured with the same apparatus, using Van Slyke's technique (23), except that lactic acid replaced sulfuric acid as the reagent

TABLE IV.

Previous Measurements of Heat of Reaction.

(1) Author.	(2) Hemoglobin preparation.	(3) Temperature.	(4) Temperature rise.	(5) Gas absorbed.	(6) Heat developed per gm. Hb.	(7) Heat developed per gram molecule Hb.	(8) Heat of solution calculated.	(9) Average molal heat of reaction.
				Oxygen + hemoglobin.				
		°C.	°C.	vol. per cent	calories	calories	calories	calories
Berthelot (14).	Defibrinated sheep blood.	9	0.115	20.2	0.895	14,960	3,500	11,640
			0.108	18.3	0.918	15,320		
Torup (15).	Crystalline horse Hb.	17	0.050	4.8	0.754	12,600	—	11,300
			0.042	3.7	0.658	11,000		
			0.041	3.7	0.623	10,400		
			0.046	4.6	0.678	11,300		
Barcroft and Hill (16).	Crystalline dialyzed dog Hb.	16	0.138	11.9	1.82	30,400	3,300	27,600
					1.98	33,070		
					1.75	29,230		
Du Bois-Reymond (17).	Defibrinated horse blood.	20	0.17	19	1.06	17,700	3,200	19,650
			0.16	21	1.09	18,200		
			0.32	34	1.21	20,200		
			0.11	11	1.33	22,200		
			0.24	23	1.39	23,200		
			0.34	26	1.73	28,900		
			0.30	23	1.77	29,600		
		43	0.08	10	0.97	16,200	2,400	13,800
	Crystalline horse Hb.	20	0.04	6.7	0.80	13,400	3,200	13,800
			0.12	16.2	0.99	165,00		
			0.02	2.3	1.14	19,000		
			0.02	2.3	1.14	19,000		
	Defibrinated horse blood. Only partially reduced.	20	0.04	5	1.05	17,500	3,200	15,100
			0.08	10	1.05	17,500		
			0.09	10	1.20	20,000		

TABLE IV—*Concluded.*

(1) Author.	(2) Hemoglobin preparation.	(3) Temperature.	(4) Temperature rise.	(5) Gas absorbed.	(6) Heat developed per gm. Hb.	(7) Heat developed per gram molecule Hb.	(8) Heat of solution calculated.	(9) Average molal heat of reaction.
Carbon monoxide + hemoglobin.								
		°C.	°C.	*vol. per cent*	*calories*	*calories*	*calories*	*calories*
Berthelot (14).	Defibrinated sheep blood.	9	0.098		1.08	18,030	3,400	15,300
			0.125		1.16	19,300		
Du Bois-Reymond (17).	Defibrinated horse blood.	38	0.17	19	1.2	20,000	2,100	17,800
			0.23	33	0.9	15,000		
		20	0.19	22	1.2	20,000	2,900	
			0.20	17	1.6	26,700		
			0.09	10	1.2	20,000		

to avoid the precipitation of hemoglobin. Frequent analyses of evacuated samples show that the average amount of carbon dioxide present was less than 2 volumes per cent.

Any fall in temperature due to the expulsion of carbon dioxide was automatically corrected for in the graphical method of calculating the cooling (Fig. 4), since the fall is continued after the oxygenation is complete. Any heat production due to internal metabolism of blood, such as Meyerhof (19) measured, was also compensated by the correction. A third error similarly cared for was the evaporation of octyl alcohol. Evaporation of water was prevented by the complete saturation of the gases going into the calorimeter; even failing this it was likewise offset by the method of experimentation.

Reaction of Oxygen with Hemoglobin Solutions.—All the complete measurements with oxygen are given in Table V.

The average result for 35 measurements at 22°C. turned out to be +10,050 calories per gram molecule of oxygen, standard deviation ±2,320 calories (22 per cent). When grouped statistically, these data give a slightly skewed probability curve with a mode at +11,200 calories. The heat of solution of this quantity of oxygen was calculated to be +3,100 calories, leaving +6,950 calories as the average heat of reaction at 22°C.

A smaller number of measurements were made at 38°C. The average result for these eight determinations was +8,050 calories per gram molecule of oxygen, ±1,700 calories (21 per cent). The heat of solution at this temperature was +2,700 calories, leaving +5,350 calories as the average heat of reaction at 38°C.

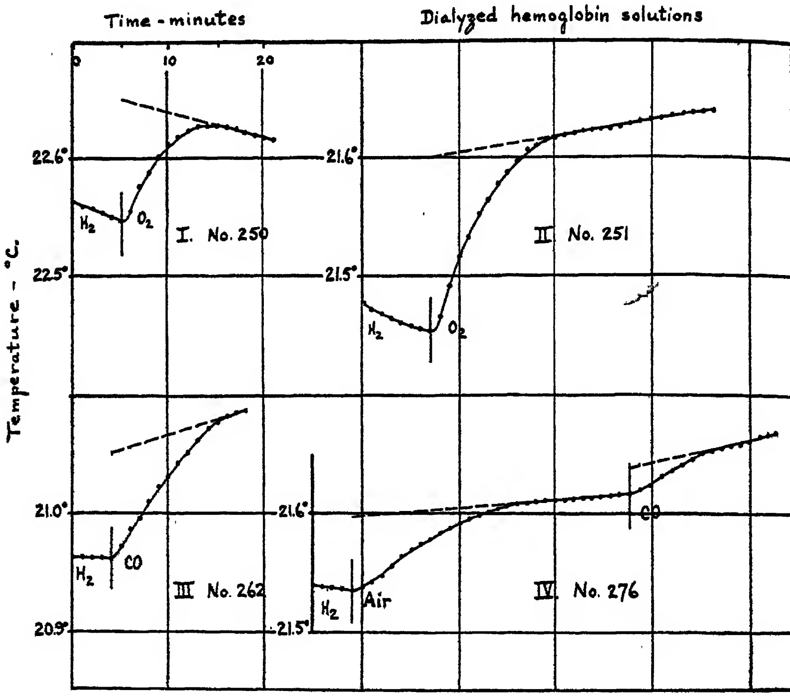

FIG. 4. Temperature changes recorded during thermochemical experiments with hemoglobin solutions.

For both temperatures together, the average was +6,650 calories.

The lower value at body temperature as compared to room temperature, while within the limit of error, is in agreement with the principles of thermodynamics. A smaller value of Q at body temperature was also obtained by du Bois-Reymond (17).

It should be noted that the amount of heat produced when hemoglobin unites with oxygen is not definitely correlated with

TABLE V.

Heats of Reaction of Oxygen with Hemoglobin.

(1) No.	(2) Solution.	(3) Temperature rise.	(4) Solution.	(5) Heat capacity.	(6) Initial HbO_2.	(7) Final HbO_2.	(8) Heat evolved per gram molecule HbO_2.
		22°C.					
		°C.	*cc.*	*calories/degree*	*per cent*	*per cent*	*calories*
236	Dialyzed whole blood.....	0.073	100	106		13.3	10,700
237	" corpuscles......	0.050	100	105		17.5	5,400
238	" "	0.056	100	106		13.0	8,900
238a	" "	0.070	97	102		15.3	8,700
239	" whole blood.....	0.065	95	101	1.2	13.5	9,400
240	" " "	0.059	94	99	3.7	13.4	10,700
244	Corpuscles + 0.9 per cent NaCl.	0.060	97	96	11.2	29.9	5,300
245	Dialyzed corpuscles......	0.099	97	99	6.4	22.1	10,400
247	" "	0.118	89	92	0.9	18.5	11,600
248	Corpuscles + 0.9 per cent NaCl.	0.254	97	93	0.6	37.3	11,100
249	Dialyzed corpuscles......	0.078	97	105	0.8	11.3	13,400
250	" "	0.103	97	103	1.4	17.1	11,600
251	" "	0.145	97	99	1.1	23.3	11,100
252	" "	0.100	97	103	1.6	17.0	11,500
253	" "	0.143	97	102	1.3	19.2	14,000
254	Corpuscles + 0.9 per cent NaCl.	0.148	100	100	3.1	23.0	12,400
255	Whole blood.............	0.080	95	99	2.9	19.1	8,600
256	Dialyzed corpuscles.......	0.105	97	103	0.9	17.1	11,500
259	" " + 0.002 M KH_2PO_4........	0.058	97	103	1.0	16.3	6,700
262	Dialyzed corpuscles + 0.003M KH_2PO_4.........	0.076	97	104	1.1	12.1	12,800
263	Dialyzed corpuscles......	0.114	104	110	1.0	16.4	13,000
264	" " + 0.074 N $NaHCO_3$........	0.074	70	74	2.3	14.5	11,600
265	Dialyzed corpuscles + 0.068 N $NaHCO_3$........	0.045	89	96	1.7	12.0	7,900
276	Dialyzed corpuscles......	0.063	96	101	8.5	18.4	11,200
278	" "	0.070	97	103	1.4	15.7	8,700
281	" "	0.086	97	103	1.0	16.0	10,000
281a	" " + 0.060 N KOH	0.075	97	102	2.5	15.4	10,200
283	Dialyzed corpuscles......	0.079	94	99	2.0	15.1	10,600
284	" "	0.100	88	89	5.7	22.3	10,200

TABLE V—*Concluded.*

(1) No.	(2) Solution.	(3) Temperature rise.	(4) Solution.	(5) Heat capacity.	(6) Initial HbO_2.	(7) Final HbO_2.	(8) Heat evolved per gram molecule HbO_2.
	22°C.—Concluded.						
		°C.	*cc.*	*calories/degree*	*per cent*	*per cent*	*calories*
286	Dialyzed corpuscles + 0.046 N lactic acid......	0.109	102	104	1.6	20.7	10,100
287	Dialyzed corpuscles......	0.132	88	90	1.4	21.6	11,200
288	" " + 0.046 N lactic acid......	0.076	99	106	4.2	14.6	13,100
289	Whole blood.............	0.048	95	103	5.0	13.7	9,900
289a	" "	0.026	97	104	4.6	14.8	4,600
290	" "	0.080	94	100	5.3	15.6	13,800
	38°C.						
293	Dialyzed corpuscles.....	0.094	98	102	0.3	20.8	7,800
293a	" "	0.049	95	99	13.5	20.8	11,700
294	" "	0.064	67	71	1.9	19.3	6,500
294a	" "	0.074	85	89	5.5	19.0	9,600
296	" "	0.081	97	103	1.0	18.0	8,400
296a	" "	0.066	97	103	1.1	18.8	6,600
296b	" "	0.086	92	94	1.2	23.0	6,700
297	Corpuscles + 0.9 per cent NaCl..................	0.151	96	93	1.9	36.4	7,100

Formula for calculation: $$(8) = \frac{(3) \times (5) \times 16{,}700 \times 100}{(4) \times [(7) - (6)]}$$

the presence of salts, acids, alkalies, or other proteins. Moreover, the age, dilution and previous treatment of the solutions, and substitutions of air for pure oxygen have no controlling influence. The latter portion of the oxygenation evolves as much energy as the total oxidation; the strict proportionality between combined oxygen and temperature production is shown in Fig. 5.

The heat of solution of the oxygen merely dissolved in blood is negligible, since the solubility of oxygen is so small that no heat of solution in water could be demonstrated directly. Moreover, it is nearly compensated by the displacement of hydrogen from solution.

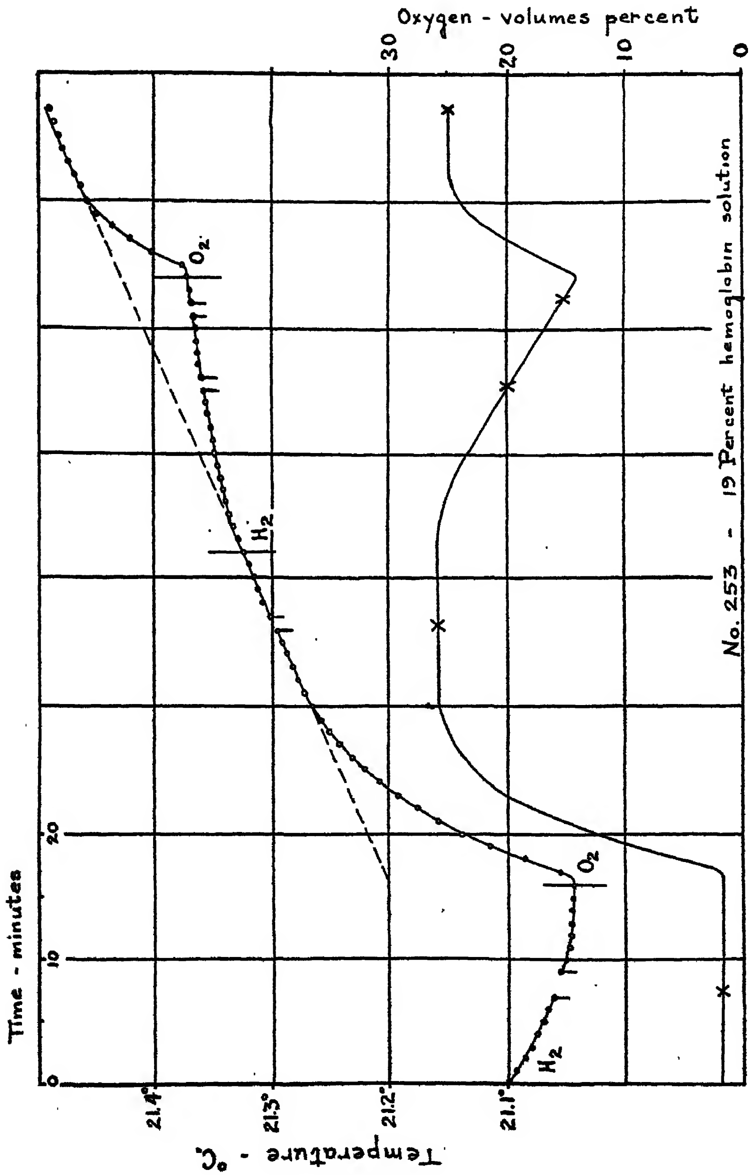

FIG. 5. Changes in temperature and in oxygen saturation during an experiment with hemoglobin.

TABLE VI.

Heat of Reaction of CO with Hemoglobin.

(1) No.	(2) Solution.	(3) CO mixture used.	(4) Temperature rise.	(5) Solution.	(6) Heat capacity.	(7) Initial HbO_2.	(8) Final HbCO.	(9) Heat developed per gram molecule HbCO.	(10) Average heat of reaction.
	Evacuated.								
			°C.	cc.	$\frac{calories}{degree}$	per cent	per cent	calories	calories
266	Corpuscles + 0.9 per cent NaCl.	Illuminating gas.	0.108	87	90	1.6	16.2	12,800	
267	Dialyzed corpuscles.	" "	0.127	85	89	0.9	16.0	14,700	
269	Whole blood.	" "	0.165	94	97	3.8	16.9	21,700	
272	Dialyzed corpuscles.	CO from oxalic + sulfuric acid.	0.054	92	100	1.5	5.0	28,000	
274	Whole blood.	CO from formic + sulfuric acid.	0.060	69	72	4.6	14.0	11,100	14,700
275	Corpuscles + 0.9 per cent NaCl.	CO from formic + sulfuric acid.	0.152	94	97	2.5	16.5	18,700	
277	" + 0.9 " " "	CO from formic + sulfuric acid.	0.140	97	100	6.8	17.9	21,700	
282	Dialyzed corpuscles.	CO from formic + sulfuric acid.	0.088	97	102	1.5	15.1	11,350	

Oxygenated.									
276	Dialyzed corpuscles.	CO from formic + sulfuric acid.	0.022	94	98	18.4	18.4	2,080	
283	" "	CO from formic + sulfuric acid.	0.030	91	96	15.1	15.1	3,500	
288	" "	CO from formic + sulfuric acid.	0.024	91	95	14.6	14.6	2,860	3,000
297	Corpuscles + 0.9 per cent NaCl.	CO from formic + sulfuric acid.	0.083	90	92	36.4	36.4	3,700	

Formula for calculation: $(9) = \frac{(4) \times (6) \times 16{,}700 \times 100}{(5) \times [(8) - (7)]}$

The standard deviation of 22 per cent in these measurements is perhaps as significant as the magnitudes themselves. The preliminary experiments with inorganic reactions have indicated that an accuracy of about 1 per cent can be attained. That such an agreement was not obtained with whole blood, and that previous workers on this problem have not agreed, indicates that important influences still uncontrolled are at work.

The result obtained for the heat of reaction of oxygen with hemoglobin, approximately +7,000 calories, is lower than that obtained by Berthelot (14), Torup (15), Barcroft and Hill (16), or du Bois-Reymond (17).

Reaction of Carbon Monoxide with Hemoglobin Solutions.—Only a few measurements of the heat developed in this reaction were made, using gas generated from formic acid and sulfuric acid. Similar results were obtained with illuminating gas, which is composed, chiefly of hydrogen, carbon monoxide, and hydrocarbons (Table VI). In all cases hydrogen was used to give a fore period (Fig. 4, Curve III). Control measurements with water showed no appreciable heat of solution.

The average value for eight measurements was +17,500 calories per gram molecule, ±5,600 calories (32 per cent). The heat of solution of a gram molecule of carbon monoxide is +2,800 calories, leaving +14,700 calories. This is very close to the result of Berthelot (14).

A series of four experiments in which carbon monoxide replaced oxygen gave a smaller development of heat, averaging +3,000 calories. The heats of solution of oxygen and carbon monoxide in this case compensate each other. If the comparative values for oxygen and carbon monoxide, +7,000 and +14,700 calories are correct, it is to be expected that +7,700 calories will develop when carbon monoxide replaces oxygen. That only +3,000 calories were evolved, indicates the importance of other factors which are either experimental or chemical.

Reaction of Carbon Dioxide with Defibrinated Blood.—The amount of energy liberated when carbon dioxide combined with solutions of dialyzed hemoglobin varied tremendously, depending upon the ionic equilibria of the solution. But when carbon dioxide combined with whole blood the results were remarkably uniform, even with the blood of different individuals. The course

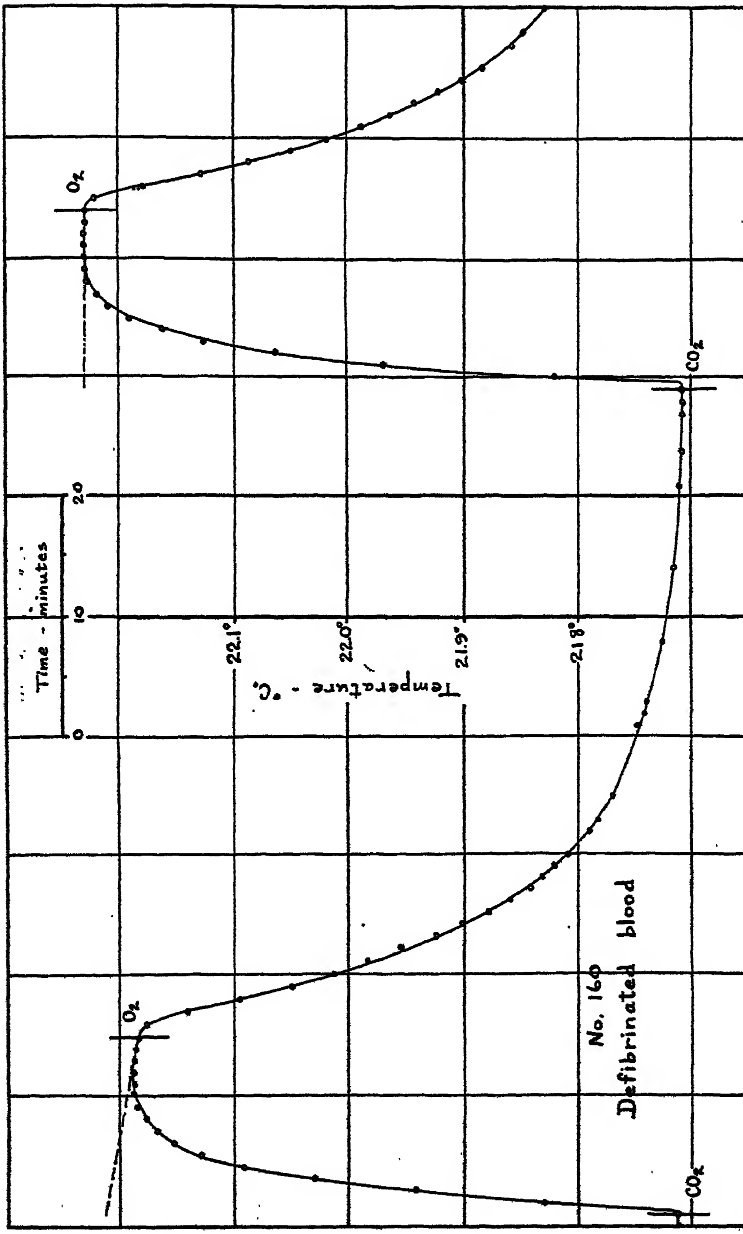

FIG. 6. Temperature changes during a thermochemical measurement using carbon dioxide and beef blood.

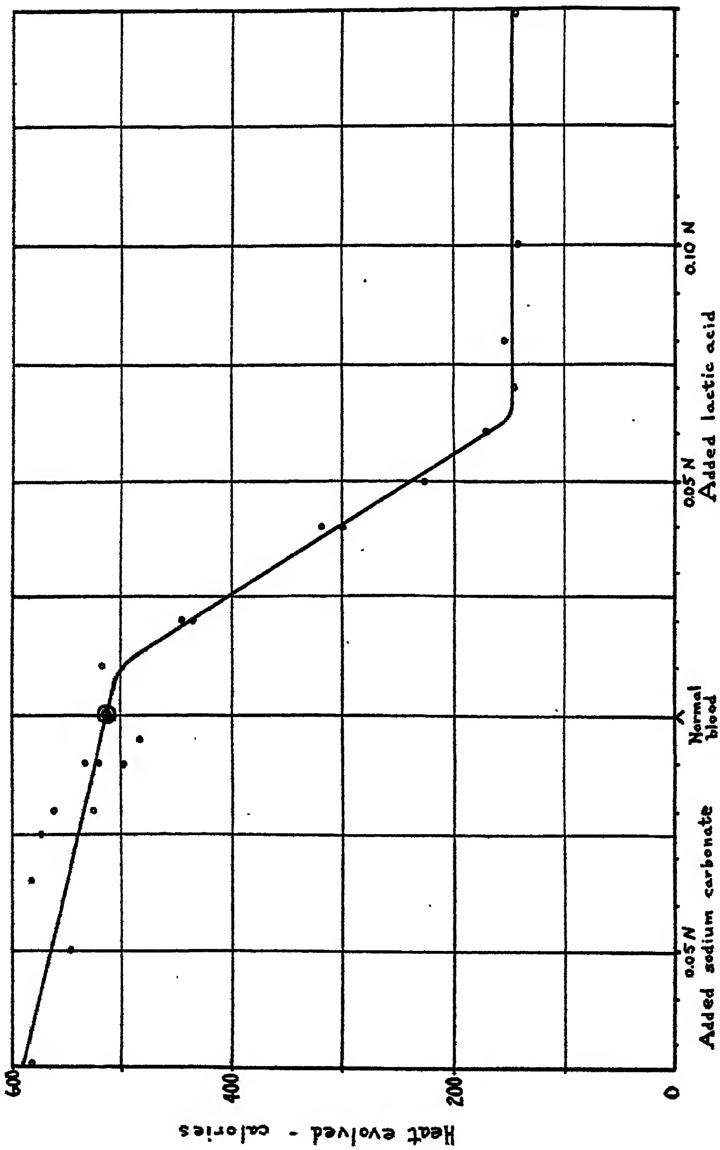

FIG. 7. Heat evolved by the saturation of 1 liter of beef blood with carbon dioxide at 22°C. The point for normal blood is an average of twenty-eight measurements.

of a typical experiment is given in Fig. 6. The blood was usually laked, octyl alcohol being present.

A series of twenty-eight measurements in which carbon dioxide was passed into fully oxygenated blood gave an average result of +513 calories per liter (standard deviation ±12 per cent). The defibrinated beef blood used included samples from eight different animals. The reverse reaction in ten measurements averaged −476 calories. When the blood was evacuated and saturated with hydrogen, the passage of carbon dioxide produced +486 calories per liter in six determinations.

Interesting results were obtained by adding known amounts of lactic acid or sodium carbonate to the blood; then passing carbon dioxide into it. The data are plotted in Fig. 7. It will be seen that 0.065 N lactic acid was sufficient to exhaust the acid-combining power of the blood. Added carbonate increased the heat production in strict proportion to the available alkali.

DISCUSSION.

The first worker to measure the heats of reaction of hemoglobin compounds, Berthelot (14), was interested in locating the heat production of the animal body. When he found that oxygen liberated considerable energy in combining with hemoglobin, he supposed that much heat was produced in the lungs, and calculated this to be about one-seventh of the human body's energy output. He suggested, however, that most of this portion of energy was used in the lungs in vaporizing water. Berthelot supposed that the liberation of carbon dioxide in the lungs involved little or no energy exchange.

From the results of the present experiments the heat exchange in respiration can be calculated. In saturating 1 liter of blood, carbon dioxide liberates 513 calories; oxygen liberates 84 calories. Complete saturation with oxygen requires an amount of gas which is only one-seventh of the carbon dioxide required for saturation, so that when the respiratory quotient is 1 these two reactions compensate almost exactly in chemical energy change. In the living body this is approximately true both in lungs and in tissues.

The dissociation equilibrium of oxyhemoglobin was first represented mathematically by Hüfner (24). Various discrepancies

between theory and fact have presented themselves, which have led to modifications of the Hüfner equation: $K = \frac{[Hb]\,[O_2]}{[HbO_2]}$. Nevertheless, the data of Barcroft and Roberts (25) showed that solutions of hemoglobin can be obtained for which K is constant at a given temperature for all oxygen tensions.

The data of Bert (26) and of Hüfner (27) show that the dissociation of oxyhemoglobin in whole blood increases with temperature. Barcroft and King (28) have plotted the actual curves at several temperatures, not only for blood but also for dialyzed hemoglobin solutions, and it is readily observed in their data that K, as defined above, is many times greater at 38°C. than at 14°C. The measurements of Barcroft and Hill (16) show the same change in dissociation with temperature.

Henri (29) was the first to suggest that by means of the isochore a relationship might be found for oxyhemoglobin between the temperature coefficient of K and the heat of reaction, Q. He showed that the heat of reaction ought to be very large, since K increases sixfold for a temperature change of 20°. Barcroft and Hill (16) again calculated this relationship, and found that Q must have a value of approximately 28,000 calories. These authors considered both K and Q as factors in a heterogeneous equilibrium; *viz.*, between gaseous oxygen and dissolved hemoglobin. In this paper their data are recalculated in terms of dissolved oxygen, corresponding to the form of our own data.

Calculations based upon the present series of experiments yield an average value for Q of roughly 7,000 calories. This series includes 43 determinations, as contrasted with 24 determinations by four other authors. Individual determinations in this series vary from 1,500 to 10,900 calories, while the results of others vary from 10,200 to 29,870 calories.

In Fig. 8 the variation of K with temperature is plotted, assuming that Q is 7,000 calories. In the same graph is plotted the experimental variation of K as found by Barcroft and Hill (16). In the first case K increases 1.4 times for 10°, in the second case 3.1 times. K is plotted here in arbitrary units, which represent approximately its true value when oxygen concentration is calculated in gram molecules.

It is evident that the experimental values of K and Q are not related by the simple isochore of van't Hoff. Careful criticism has revealed errors in the technique neither of our measurements of Q nor of Barcroft's (16, 28) measurements of the temperature coefficient of K which are sufficiently great to explain away the discrepancy. It is, therefore, a real one.

It seems worth while to recognize a number of unmeasured factors in hemoglobin systems. The measured value of Q on the

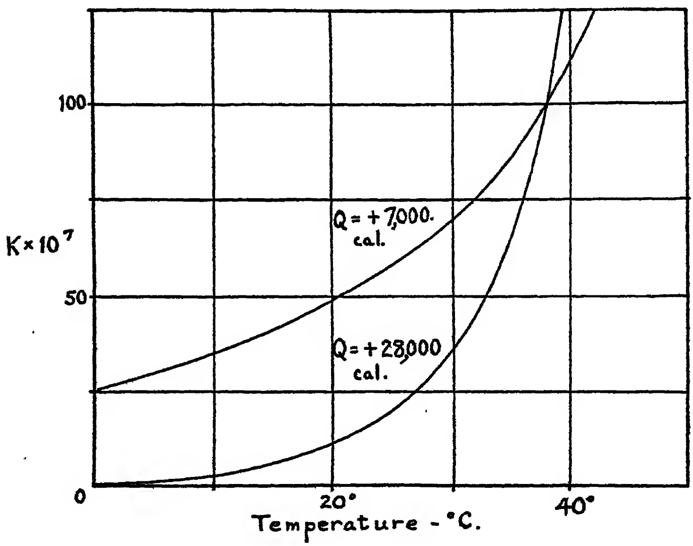

Fig. 8. Variation with temperature of the equilibrium constant for the dissociation of oxyhemoglobin, as calculated by van't Hoff's isochore.

one hand, undoubtedly includes energy derived from the aggregation and ionic dissociation of hemoglobin, at least one and perhaps both of which occur during oxygenation. The measured value of the temperature variation of K, on the other hand, probably includes the following factors:

1. Change in the solubility of oxygen with temperature. This is the only one of the factors which can be corrected for, and when calculations are applied to Barcroft's (16, 28) data, they are altered relatively slightly.

2. Change in the aggregation equilibria of hemoglobin and of oxyhemoglobin. While Hill (30) and Haldane (31) have been able to calculate the amount of aggregation of molecules which may take place when hemoglobin gives up oxygen, it is not known how this ratio of equilibrium constants varies with temperature.

3. Change in ionic dissociation of hemoglobin and of oxyhemoglobin. The deductions of Henderson (32) and the experiments of Adolph and Ferry (20) show that probably the ionization of hemoglobin changes its equilibrium with variation in combined oxygen. It is impossible to distinguish this change from the changes due to other shifts in ionic equilibria, all of which are probably influenced greatly by temperature.

4. The redistribution of cations associated with hemoglobin and with oxyhemoglobin. There is little doubt that each salt of either substance differs in its behavior toward oxygen.

5. The redistribution of all ions and molecules not chemically related to hemoglobin or oxyhemoglobin. This is at a minimum in dialyzed preparations. Barcroft (33) has drawn the conclusion that there are at least two ways in which the marked influence of salts is exerted; first, by changing the aggregation of hemoglobin, and secondly, by changing the ionization of hemoglobin. There may in addition be a direct influence of salts.

In the light of these considerations it is perhaps not surprising that simple theory and complex fact are apparently at variance. The discrepancies may be summarized for the present in the statement that the active mass of hemoglobin differs from its measurable properties in its behavior toward oxygen.

It is of interest to apply the isochore to the equilibrium between carbon monoxide and hemoglobin. The equation $k = \frac{[Hb]\ [CO]}{[HbCO]}$ has been shown by Haldane (31) and Hartridge (34) to be subject to the same conditions as that for oxyhemoglobin. By measuring the equilibrium when both oxygen and carbon monoxide are present in solution at several different temperatures, they showed that the change with temperature in the k for carbon monoxide is from 5 to 10 per cent higher than in the K for oxygen.

Applying the isochore to their data one would expect to find q for carbon monoxide hemoglobin approximately 8 per cent greater

than Q for oxyhemoglobin, or about 7,500 calories, if our average for Q is correct. Our measurements of q, in common with those of Berthelot (14) and of du Bois-Reymond (17), show that the actual q is very much larger than the heat of reaction for oxygen, about 15,000 calories. Reversing the use of the isochore, it is found that k increases rapidly with temperature in such a case. Moreover, the experiments in which carbon monoxide replaced oxygen which was already combined with hemoglobin show an evolution of heat, not of 500 calories nor of 8,000 calories, but of 3,000 calories.

The influences of acids, alkalies, salts, and dilution upon the dissociation of carbon monoxide hemoglobin are the same as those upon oxyhemoglobin (31, 34). The temperature effect and the energy of reaction are the only chemical properties studied so far that are known to differ for the two compounds; and these two quantities are not correlated in the expected manner in the case of either compound.

SUMMARY.

1. The technique and apparatus used in measuring the energy exchange in gas-liquid reactions are described.

2. The thermochemical method is shown to be useful in studying (1) the location of animal heat production, (2) the velocity of reactions, (3) the amount of oxygenation and reduction of hemoglobin, (4) the neutralizing power of solutions, and (5) the heat of reaction as applied in the use of van't Hoff's isochore and in the measurement of chemical affinity.

3. It is shown that neither the isochore nor the mass law can be applied directly to the oxyhemoglobin system, under the limitation of present analytical methods. Several unmeasured factors that occur in every hemoglobin system are suggested as partially responsible for this.

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THE PHYSIOLOGY OF THE PHENOLS.

I. A QUANTITATIVE METHOD FOR THE DETERMINATION OF PHENOLS IN THE BLOOD.

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Liver function and related problems have interested several workers in this laboratory during the past few years. As a part of this research program it seemed advisable to examine critically the conjugation of phenolic substances in the body. The mechanism of absorption, conjugation, distribution, and excretion can be studied when a suitable method is at hand. The method described and controlled below is sufficiently accurate to make possible a careful study of these phenolic substances in the blood and body tissues.

Benedict and Theis (1) in 1918 made an attempt to apply the urinary phenol method of Folin and Denis (3) to the blood. The principal modification consisted in a separate determination of uric acid by Benedict's method and an addition of sodium bisulfite to stabilize the color. Their conclusion from an examination of a number of pathological cases was that blood contains an average of 4.70 mg. of phenol per 100 cc., that it contains no conjugated phenols, and that the polyphenols appear to represent about one-third of the total phenols.

From all the experimental evidence presented by other workers we must regard as an established fact that the phenols are formed in the intestinal tract by bacterial decomposition of tyrosine, and that they are excreted largely in the urine. Only a small percentage is excreted with the feces (4). Also, it has been conclusively shown that a large percentage of the urinary phenols exists in the conjugated form (9). Since we have no reason to believe that conjugation occurs either in the kidneys or in the bladder we may

safely assume that at some time or other the conjugated phenols pass through the blood prior to excretion. It should, therefore, be possible to demonstrate and measure these substances in the blood stream.

An examination of Benedict and Theis' method shows that there are several factors which may well prevent the demonstration of minute quantities of conjugated phenols. The separate determination of uric acid not only complicates the method considerably, but involves an unavoidable experimental error. The boiling of the blood filtrate down to less than half the volume is the process primarily responsible for their inability to demonstrate conjugated phenols in the blood. Benedict and Theis state that only 85 per cent of resorcinol added to the blood is recovered by their method and that phenol itself added to the blood disappears completely during the boiling of the filtrates. There are sufficient reasons to believe (9) that the bulk of the conjugated phenols is made up of two very volatile phenols—*p*-cresol and phenol—and it is, therefore, logical to assume that the boiling of the filtrates in Benedict and Theis' method is responsible for the disappearance of the conjugated phenols.

However, it is possible to modify Folin and Denis' original method so that it may be applied to blood. It seemed important to eliminate heat as a factor in the precipitation of the blood proteins and in the concentration of the filtrate. The precipitation of proteins with tungstic acid, as described by Folin and Wu (5) is an excellent substitute for the boiling acetic acid; the precipitation is fully as complete, and the reaction takes place at room temperature. In order to remove the last traces of protein, aluminum cream is added. It was further found that with a standard of 5 mg. of phenol per 100 cc. set at 20 in the Duboscq colorimeter, the color developed in the blood filtrate is easily and accurately readable. This eliminates the concentration of the filtrate by boiling as carried out by Benedict and Theis. Another objection to Benedict's method is the separate determination of uric acid by an entirely different and time-consuming method. In order to simplify this step a number of uric acid precipitants, such as Morris' (8) zinc salt and Curtman and Lehrman's (2) nickel salt were tried, but, while the precipitation of uric acid is complete, the excess zinc or nickel is difficult to remove from the solution, and

if not removed interferes with the final color reaction. Folin uses silver lactate in lactic acid as a precipitant for uric acid, but the difficulty with lactic acid lies in the fact that it gives a blue color with Folin's phenol reagent; a fact which was overlooked by Folin and may in part be responsible for his high figures. Nevertheless, this method of precipitating the uric acid was retained, but the error caused thereby is now corrected by addition of a corresponding amount of lactic acid to the standard. Benedict's innovation of stabilizing the color by addition of sodium sulfite

TABLE I.

*Recovery of Phenols Added to Blood.**

Before addition of phenols.	After addition of 5.81 mg. *p*-cresol† to 100 cc. of blood.
mg. per 1,000 cc.	*mg. per 1,000 cc.*
24.6	74.0
29.1	78.9
25.6	76.0
	Addition of 5 mg. phenol.
25.2	75.0

* The blood proteins must be precipitated immediately after the addition, as prolonged standing destroys a part of the phenols.

† 5.81 mg. of *p*-cresol are equal in color production to 5 mg. of phenol. The colorimeter reading is expressed as phenol.

TABLE II.

Accuracy of Separate Simultaneous Determinations of Phenol.

Determination.	Dog 1.	Dog 2.	Dog 3.
First............	28.4	36.25	31.0
Second..........	28.75	37.1	30.6
Third...........	28.0	37.50	

is omitted. Due to the more complete precipitation of the proteins and the higher dilution in which the readings are made, the dirty green tinge which sometimes occurs with Benedict's method does not appear. Finally another change has been made in the method: due to the higher dilution no addition of water to make up to 50 or 100 cc. as in Folin's or Benedict's methods is necessary, and there is substituted a manipulation of the final filtrate in graduated test-tubes which greatly increases the accuracy of the method and makes possible the detection of conjugated phenols in very minute quantities.

Method.

The entire method is very simple and rapid. 10 cc. of blood are added to 50 cc. of distilled water in a 100 cc. Erlenmeyer flask. Then 10 cc. of 10 per cent sodium tungstate and 10 cc. of $\frac{2}{3}$ N sulfuric acid are added, the flask is closed with the thumb or rubber stopper, and vigorously shaken for a few seconds. To precipitate the proteins completely 10 cc. of aluminum cream are added and the flask is again shaken. The contents are transferred to a 100 cc. centrifuge tube and centrifugalized for 45 minutes. The supernatant fluid is filtered to the 45 cc. mark in a narrow 50 cc. graduate, 5 cc. of a 5 per cent solution of silver lactate in 5 per cent lactic acid are added, and the graduate is well shaken for 1 minute. After centrifugalization and filtration, the filtrate is ready to be examined for phenols. This last step is carried out as follows: only two narrow test-tubes are required —one graduated at 15 cc. and the other at 10 cc.—in which both the total and the free phenols are determined. Thus, any error due to the graduation of two sets of test-tubes is avoided. The procedure for the determination of *free* phenols is this: the 15 cc. tube is filled to the mark with the filtrate, 1 cc. of the phenol reagent[1] is added, and the tube is shaken. The excess silver precipitates out, the solution is filtered to the mark into the 10 cc. tube, and 5 cc. of 20 per cent sodium carbonate are added. This solution is now transferred to another test-tube in which the color develops to its maximum in about 20 minutes. The two graduated test-tubes are meanwhile used for the determination of *total* phenols. The 15 cc. tube is again filled to the mark with the same filtrate, 5 drops of concentrated HCl are added, and the tube is placed in a water bath at 100°C. for 10 minutes. Boiling of the contents of the tube is avoided and no loss of volatile phenols occurs, as was shown by repeating the determination with known amounts of phenol. If the tube has a diameter of 14 to 15 mm.,

[1] Bell's modification of the reagent (Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 508) is used, since the HCl which it contains is needed for the precipitation of the excess silver. It contains: 100 gm. of sodium tungstate, 20 gm. of phosphomolybdic acid, 50 cc. of phosphoric acid 85 per cent, 100 cc. of concentrated HCl. This is gently refluxed for 2 hours with 750 cc. of water, and at the end of the period of heating made up to 1,000 cc.

TABLE III.

Removal of Uric Acid by Precipitation.

Before addition of uric acid.	After addition of 5 mg. of uric acid to 100 cc. of blood.
Total phenols per 1,000 cc.	Total phenols per 1,000 cc.
mg.	*mg.*
23.5	23.8
25.5	25.5
29.0	29.5

TABLE IV.

Influence of Amino-Acids Normally Present in the Blood.

Before addition of casein digest.	After addition of casein digest (20 mg. of amino N to 100 cc.)
Total phenols per 1,000 cc.	Total phenols per 1,000 cc.
mg.	*mg.*
34.2	36.8
31.4	35.0
24.6	27.5

TABLE V.

Demonstration of Conjugated Phenols in Human and Dog's Blood by the Author's Method.*

Blood.	Total.	Free.	Conjugated.	
	mg. per 1,000 cc.	*mg. per 1,000 cc.*	*mg. per 1,000 cc.*	*per cent*
Human.				
Decompensated heart.....	31.2	28.5	2.7	8.6
Fracture of arm..........	37.2	33.2	4.0	10.7
Normal..................	42.8	38.0	4.8	11.2
Dog.				
Normal..................	29.4	28.2	1.2	4.1
	32.5	30.1	2.4	7.4
	38.1	35.2	2.9	7.6
	34.0	32.5	1.5	4.4
	27.4	24.4	3.0	10.9

* This table is not intended to give average values for human blood (for which purpose a much greater number of determinations must be made) but merely to demonstrate the presence of conjugated phenols.

the volume of the contents on cooling at the end of exactly 10 minutes is back to the graduation mark, so that no adjustment of volume is necessary. Then 1 cc. of the phenol reagent is added and the solution is treated in the same way as in the determination of free phenols. The difference between the total phenols and the free phenols represents conjugated phenols.

The standard is prepared as follows: 5 cc. of the stock solution of resorcinol (Benedict and Theis, 1), containing 5.81 mg. are placed in a 100 cc. volumetric flask, 0.5 cc. of concentrated HCl and 10 cc. of the silver lactate-lactic acid solution added, centrifugalized or filtered, and the filtrate is manipulated in the graduated test-tubes in the same manner as the blood filtrate in the determination of free phenols.

Recently there have been published several criticisms of Folin's phenol reagent (6, 7, 9), from which it appears that this reagent gives a blue color with a great many substances, and that it is by no means specific for phenols and the closely allied hydroxy-acids. It must be said, however, that most of these substances are normally not present in the blood, and that the normal constituents of the blood which interfere with the reaction can either be readily removed (uric acid, Table III), or are in such small concentration (amino-acids, Table IV), that their presence accounts, at best, for only a negligible fraction of the color developed. Further, in experiments such as reported in the later papers of this series, where we are dealing with the measurement of injected or ingested phenols, the color-producing effect of these interfering substances is entirely eliminated by a preliminary determination.

SUMMARY.

1. A method is described for the determination of phenolic substances in blood, which is based on Folin's method for the determination of phenolic substances in urine.

2. Contrary to the opinion of Benedict and Theis (1) conjugated phenols are present in human as well as in dogs' blood and can be demonstrated with the above method.

I am indebted to Professor W. R. Bloor and Dr. C. L. A. Schmidt of the Department of Biochemistry for their helpful advice and criticism in the development of this method.

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THE PHYSIOLOGY OF THE PHENOLS.

II. ABSORPTION, CONJUGATION, AND EXCRETION.

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With a suitable method at hand for the quantitative estimation of phenolic substances in the blood we took up a study and review of the factors concerned in the normal and abnormal metabolism of phenols in the body, directing particular attention to the liver. Many investigators have studied this question and have marshalled experiments to prove or disprove that the conjugation of phenols takes place in the liver, or again in the kidney, or elsewhere in the body. The best evidence to date is in favor of conjugation taking place in the liver, but in the face of contradictory experiments it must be admitted that much of this evidence is indirect and the question is at least open to debate. We may now review some of the recent work which concerns our thesis.

Baumann (1) noted a temporary accumulation of conjugated phenols in the liver of a dog poisoned with phenol, and showed that he could obtain nineteen times more phenol-sulfuric acid from that organ than from other organs or the blood. He suggested that the liver is the organ primarily concerned with the synthesis. Christiani and Baumann (4) tied off the ureters of a dog and showed that no accumulation of ethereal sulfates occurs in the blood. From this they concluded that, at least when the ureters are tied, the kidneys take no part in the conjugation of phenols. In other and more convincing experiments they ligatured all renal vessels and poisoned the dog with phenol. Synthesis occurred to the same extent as with normal dogs. From these experiments they concluded that if the kidneys are at all concerned with this reaction they are concerned with it to a negligible extent only. In the pursuit of the question of the participation of the kidneys in this synthesis Baumann and Herter (2) had already tried to perfuse the kidneys with blood containing phenol and sodium sulfate and had been unable to show any synthesis. Kochs (14) ground up liver, kidney, pancreas, and muscle, and added phenol plus sodium

sulfate. He states that a moderate amount of conjugation was demonstrable in each case, and that negative results were obtained with thymus gland. Landi (15) repeated Kochs' experiments but could find no conjugation. From perfusion experiments with some of the organs he concludes that the intestine is the seat of the synthetic process. Lang (16) found small amounts of conjugated phenols in the urine of geese whose livers he had extirpated, and thinks that the liver is not the exclusive, although the most important, organ of conjugation. Herter and Wakeman (11) added equal amounts of phenol to blood and ground brain, muscle, kidney, and liver, and showed an increasing disappearance of phenols in the order given. They do not believe, however, that this disappearance is due to a conjugation with sulfuric acid; rather they favor a chemical destruction or a "loose combination of the phenol molecules with the molecules of the substance." Salta (19) thought that he could determine the place of conjugation by an analysis of various organs for phenol-sulfuric acid. The largest amount was found in the liver. Then follow in decreasing amounts in the order given: muscles, lungs, intestine, stomach, nerves. No conjugated phenols were found in the brain. From this he reasons that all these organs play a part in the synthesis. Embden and Glaessner (6), in a number of carefully controlled perfusion experiments, show that the conjugation takes place almost exclusively in the liver, although very small amounts of ethereal sulfates were found in the lungs and kidneys.

The work of Herter and Wakeman (11) is sometimes cited (5) as proof that other organs than the liver, *i.e.*, the intestinal epithelium, kidney, muscle, brain, blood, etc., have the ability, although to a lesser extent, to conjugate phenols with sulfuric and glucuronic acids. This statement, which is not in accordance with observable facts, is based on a misapprehension of the work of the first named authors. Although their experiments showed a disappearance of phenols when known amounts were added to and left in contact for some time with ground liver, muscle, kidney, etc., there is no evidence that this disappearance is due to a synthesis of phenol-sulfuric or glucuronic acids. In fact, Herter and Wakeman themselves state[1] that "the synthesis of indoxyl potassium sulphate cannot be accomplished by extirpated cells. It also seems improbable that the dead cells convert phenol into phenol-sulphuric acid." They then attempt to show[2] that this destruction of phenols is not in the nature of an oxidative process since they were unable to recover such oxidation products of phenol

[1] Herter and Wakeman (11), p. 317.
[2] Herter and Wakeman (11), pp. 317–318.

as hydroquinone or pyrocatechol. By subjecting liver pulp to various ferment-destroying agents (alcohol, boiling water, hot air, bichloride of mercury) and finding no decrease in the behavior towards phenol, these authors consider it highly improbable that ferments play any important rôle in this disposal of phenols, and they finally speculate[3] as to the probability of some "loose combination of the phenol molecules with the molecules of the cell substance."

It should be strongly emphasized that there is little experimental evidence for the statement that other organs than the liver have this ability to conjugate phenols. If the transformation of phenols by dead tissues is due to conjugation, it should be possible to hydrolyze these conjugated phenols by the addition of

TABLE VI.

Blood and Liver Incubated with Phenols.

Blood blank.	Blood + 5 mg. phenol per 100 cc. and 2 hrs. incubation.	Same + 5 mg. $K_2S_2O_8$ per 100 cc.	Same hydrolyzed with 5 drops HCl and heat.
mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
2.8	7.6	7.4	7.45
3.2	7.9	8.2	8.1
Liver as above.			
6.6	9.8	10.0	9.4
7.25	10.5	10.1	10.6

a few drops of HCl and the application of heat, and so recover the total amount of added phenol. That this is not the case is shown in Table VI in which are recorded the results of an experiment similar to those of Herter and Wakeman. Instead of using Millon's reagent the method described in the first paper of this series was used.

Known amounts of ingested phenols cannot be completely recovered in the urine or feces, and this loss is probably due to a similar reaction by which organ pulp disposes of phenol, *i.e.*, chemical oxidation, ferment action, or Herter and Wakeman's "loose combination."

[3] Herter and Wakeman (11), pp. 319–320.

Tauber (22) fed phenol to a dog and found that as the dose was decreased. the amount of phenol so "oxidized" was increased. On feeding 240 mg. of phenol in water *per os*, he found that 53 per cent of the ingested phenol was oxidized in the body through oxalic acid to carbon dioxide. Dubin (5) found that 68.7 per cent of ingested phenol and 50.6 per cent of *p*-cresol could be recovered from the urine. When tyrosine was given, only 17.7 per cent of the amount of phenol corresponding to the amount of tyrosine administered was recovered. Friedländer (9) recovered only 25 per cent of ingested cresol from the urine of his dogs. Numerous other workers have noted the fact that ingested phenols cannot be quantitatively recovered from the excretions. Siegfried and Zimmermann (20) fed *p*-cresol to a dog and recovered both *p*-cresol and phenol, the sum of both amounting to 32 to 48 per cent of the *p*-cresol fed. Jonescu (13) administered cresols to dogs and noted that they were oxidized in the order of ascending toxicity. The percentage oxidation was as follows: *m*-cresol 50 to 53 per cent, *o*-cresol 65 to 69 per cent, and *p*-cresol 73 to 76.5 per cent. As an example of the powerful oxidation to which phenols may be subjected in the organism, Jaffé (12) has shown that the ingestion of benzene increases the urinary output of muconic acid, and suggests that this is due to the cleavage of the benzene ring into a straight chain compound. The whole subject of oxidases and phenolases is exhaustively treated in Oppenheimer's text-book (18) and need here not be further considered. ("Die Wirkung dieser Phenolasen ist neben der Oxidation, die niemals tiefgreifend ist, häufig eine Kondensation mehrerer Moleküle. Das hängt mit ihrem physiologischen Zwecke der Schaffung unlöslicher Stoffe zusammen.")

This oxidizing action on the phenols which has been noted by Tauber to increase as the amount of volatile phenol present decreased, would explain why normally there are such small amounts of conjugated phenols in the blood: the volatile phenols produced in the intestinal tract are probably to a large extent rapidly oxidized by the intestinal mucosa and the liver—and those which escape this process are *completely* conjugated by the liver. When the oxidizing mechanism is overwhelmed by large amounts of phenol, as occurs when phenol is ingested, more of that substance reaches the hepatic tissues unchanged and the relative amount of conjugated phenols is greater. Ordinarily this oxidizing mechanism is sufficient and much larger amounts of volatile phenols than are normally produced in the intestine are oxidized within a few minutes. This serves to strengthen the argument that free volatile phenols as such cannot remain in the blood stream for any considerable period.

It now appears probable that in its effort to dispose of phenol which reaches the circulation from the intestinal tract, the body

makes use of two methods: one a process of oxidation which takes place to a considerable extent in the epithelial lining of the intestinal tract and in the liver and to a lesser extent in the other organs; the other a process of conjugation with sulfuric and glucuronic acids which takes place, as we shall later show, exclusively in the liver.

As regards the nature of the phenolic substances in the blood, there is no reason to suppose that they are essentially different from those in the urine. The most important literature dealing with that subject has been summarized by Folin and Denis (8), Dubin (5), and Tisdall (23).

EXPERIMENTAL OBSERVATIONS.

It seemed necessary to control diet factors in these experiments because of the observations of Underhill and Simpson (24) to the effect that urinary phenols vary directly with the protein intake, and with an increase in output of total phenols the percentage of free phenols remains constant. Some similar observations are recorded in Table VII which gives controls for the various diets under laboratory conditions.

Dogs weighing 20 to 35 pounds were used in these experiments. Mixed diet indicates a liberal amount of mixture of table scraps including cooked meat, macaroni, potatoes, bread, and bones.

The most important volatile phenols present in the body are *p*-cresol and phenol (7) and we, therefore, limited out experiments to these two substances focussing our attention upon *p*-cresol, as we found that complete conjugation occurs with it more rapidly than with any other phenolic substance investigated. At first we injected known amounts of phenol in a watery solution into the jugular vein of the animal and analyzed for total and free phenols the blood drawn just prior to and at stated intervals after the injection. Charts A and B give typical results of some of these early experiments. These experiments show that 5 minutes after the injection of 500 mg. of *p*-cresol or phenol the "theoretical concentration" of about 500 mg. per 1,000 cc. of blood has fallen to 80 mg. per 1,000 cc. We say "theoretical" advisedly, for the disappearance of phenols from the blood stream is so rapid that, due to the time lost during the slow injection this concentration is never approximated. The dog weighed 24 pounds and we may

assume an approximate blood volume of 1,000 cc. This almost instantaneous disappearance of injected phenols is, we believe, due to two factors; a rapid and relatively uniform distribution throughout all tissues of the body, and oxidative processes also occurring throughout the body although not with equal rapidity in all tissues. The amount of *conjugated phenols* in the blood 5 minutes after the injection is only slightly greater than before injection, but it increases rapidly to a maximum about 1 hour after injection. At the end of the hour the *free phenols* have reached their pre-injection level and the conjugated phenols fall slowly (6 to 12 hours) back to their normal level.

TABLE VII.

Diet Factors and Blood Phenols.

Dog.	Total phenols per 1,000 cc. of blood.	Conjugated phenols.	Total phenols per 1,000 cc. of blood.	Conjugated phenols.
	Exclusive carbohydrate diet.		High protein diet.	
	mg.	*per cent*	*mg.*	*per cent*
21-56	26.5	7.0	39.4	8.0
21-95	23.4	5.5	42.2	7.0
21-95	24.0	6.4	45.9	8.4
21-98	31.7	7.4	38.8	6.6
21-80	25.0	8.0	43.0	7.8
	5 to 8 days fasting.		Mixed diet.	
21-98	28.1	9.0	36.8	4.0
21-105	23.5	6.3	37.9	6.1
21-80	29.0	7.0	28.2	11.0
21-80	32.4	7.4	35.0	5.8

As these curves are typical of a large number of determinations made on different dogs, it can be safely concluded from their examination that *p*-cresol is the more quickly oxidized or conjugated substance, for the fall of the free phenols back to the original level occurs sooner in every experiment.

The injection of such large amounts of highly poisonous substances invariably produces severe systemic reactions. Immediately after injection the pupils are widely dilated, the animal has convulsions and is unable to stand. In most cases the animal becomes clinically normal within 5 minutes. In some cases

animals which have previously reacted moderately to repeated injections will not survive the unit dose. These intravenous injections are always dangerous and may destroy a valuable standardized animal. Because of this difficulty we examined the

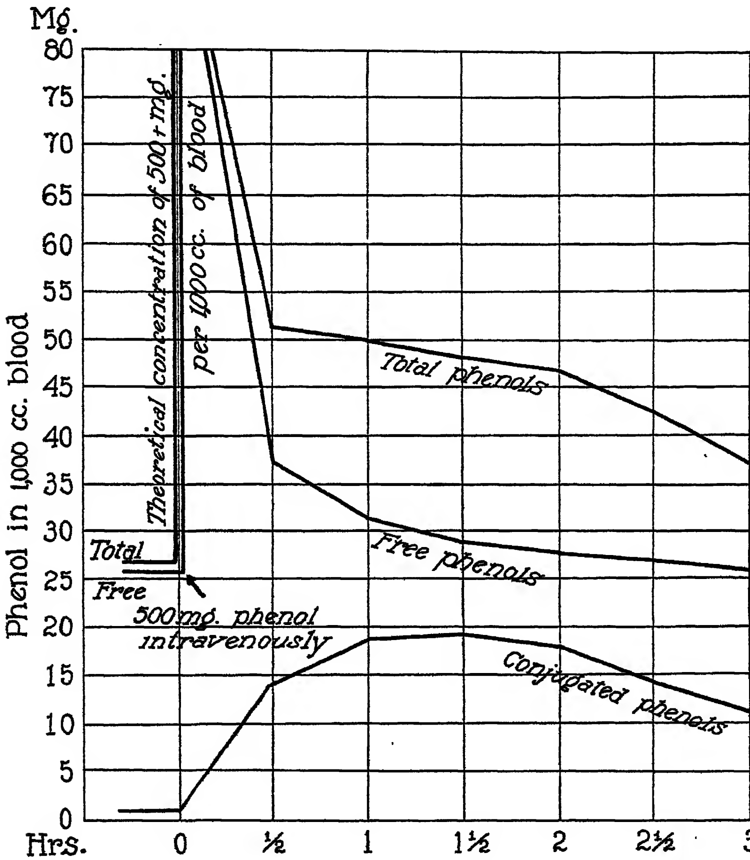

CHART A.

reaction to phenol solutions administered by stomach tube, and found that doses lethal for intravenous injection often produced no symptoms when given by stomach. An examination of Chart C shows that absorption from the intestine is sufficiently rapid and regular to be substituted in our experiments for intravenous in-

jection. In fact, the ingestion of phenols is superior to the injection as it will be seen that complete conjugation of ingested phenols is more conspicuous. This, no doubt, is due to the fact that all the ingested phenols must pass through the liver before

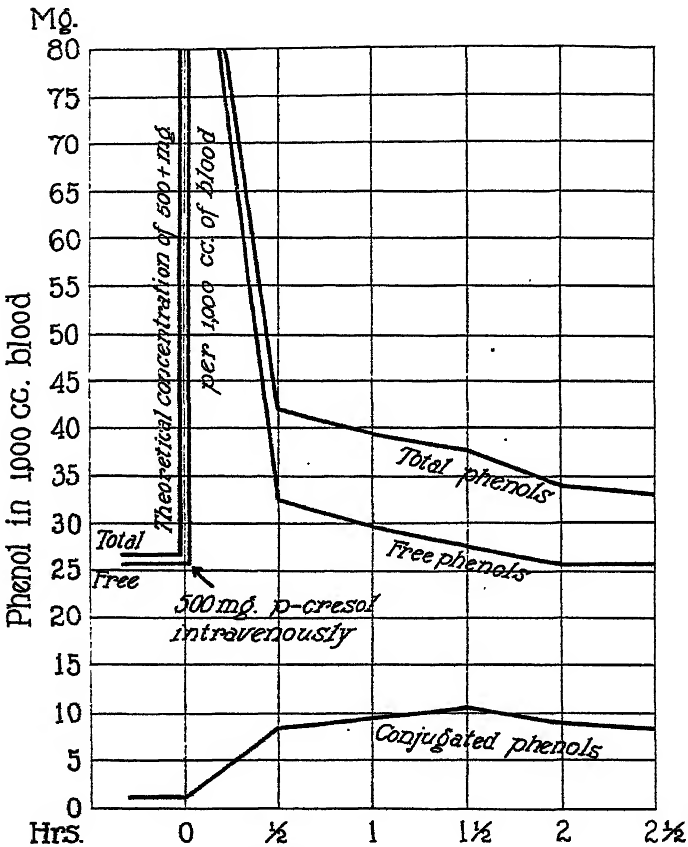

Chart B.

reaching the general circulation, while injected phenols are first distributed to all the tissues of the body and reach the liver much more slowly. The slight irregularities which sometimes occur in the ascending limb of the curve may be due to differences in stomach and intestinal absorption. In this connection it is interesting

to recall the statement of Sollmann and Hanzlik (21) that the absorption of phenols is not a steady process, because of a slowing of the local intestinal circulation by these substances. A standard dose of 50 mg. in 10 cc. of water per pound body weight given by stomach tube has been used in all experiments reported in this paper except where otherwise stated.

A closer examination of the curves obtained by plotting the results of consecutive blood analyses reveals the following: immediately after *ingestion* both the total and free phenols begin to rise—the free somewhat slower than the total. The total phenols reach their greatest concentration between ½ and 1 hour after

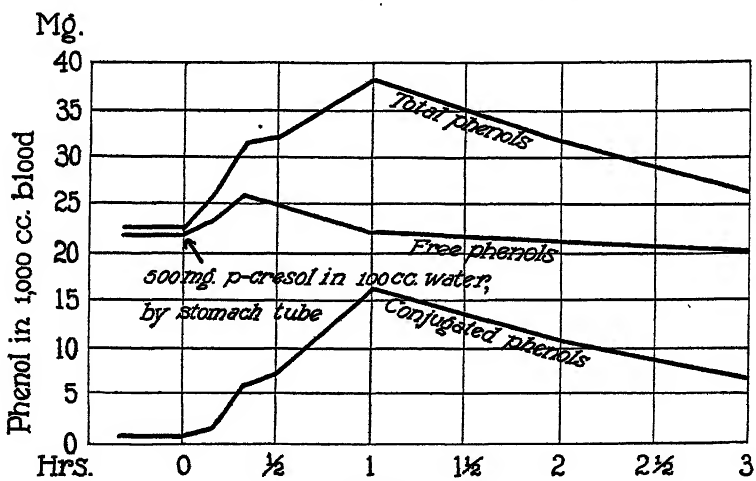

CHART C.

ingestion and after that time decline very slowly (6 to 12 hours) to their former level. The free phenols, after a slight rise within the first 20 or 30 minutes, quickly return to their old level (½ to 1 hour). In many cases the free phenols fall somewhat below that level. The resulting differences between the total and free phenols represent conjugated phenols which are plotted at the bottom of the charts. It will be seen that the conjugated phenols reach their greatest concentration 1 hour after ingestion and at this time the conjugation of ingested phenols is *complete;* an observation which corroborates Baumann (3), who showed that 1 hour after the intravenous injection of phenols the free phenols dis-

appeared from the system. After reaching the greatest concentration within 1 hour, the conjugated phenols are slowly excreted, corresponding to the slow fall of the total phenols.

From the fact that in a healthy animal the conjugation of very large amounts of ingested phenols is complete within 1 or 2 hours, it would appear unlikely that *free volatile phenols* are normally present in the blood. The conjugation of the small amount of volatile phenols produced in the intestine must be very rapid within the liver, and it is improbable that any free volatile phenols reach the general circulation under normal physiological conditions.

The bulk of the blue color (90 per cent and more) which is produced by Folin's reagent with the blood filtrate is due to substances other than true phenols. It may be due, among others, to hydroxy-acids and unidentified protein decomposition products as well as to carbohydrates and related substances (10, 17); the remainder of 10 per cent or less is produced by the conjugated phenols.

These three tables (Tables VIII, IX, and X) supplement each other and bring out several interesting points. The various organs compared with blood (Table VIII) contain substances which react with the phenol reagent and give larger figures for their "phenol" content. We have no reason to suppose that these reacting substances are true phenols.

The injection of phenol (Table IX) gives an immediate increase in phenol content of the blood and tissues. There is probably a pretty uniform distribution of the phenols in the blood and tissues but we record in analyses about 40 mg. increase in blood and 25 to 30 mg. increase in the parenchymatous organs.

After an hour's interval following a phenol injection (Table X) we note a uniform distribution of conjugated phenols in the blood and organs.

From these facts we wish to assume that free phenols, when injected into the blood stream, are distributed throughout the living tissues. After conjugation by the liver they again diffuse out to the tissues before being excreted (Table X). When phenols are given by stomach no such distribution of *free phenols* occurs because they are conjugated by the liver before reaching the general circulation. This last fact explains why doses of phenol,

lethal when injected into the blood stream, show no effects when given by stomach tube, but are lethal once again when administered by stomach tube to a dog whose liver has been seriously injured.

TABLE VIII.

Distemper Dog, Killed with Chloroform.

Organ.	Total phenols per 1,000 gm.	Free phenols per 1,000 gm.	Conjugated phenols.
	mg.	*mg.*	*per cent*
Blood	22.3	20.8	1.5
Kidneys	65.8	65.8	0.0
Liver	71.3	70.1	1.2
Spleen	59.4	57.9	1.5

TABLE IX.

Dog Killed by Injection of Phenol into Jugular Vein, Death 5 Minutes after Injection.

Organ.	Total phenols per 1,000 gm.	Free phenols per 1,000 gm.	Conjugated phenols.
	mg.	*mg.*	*per cent*
Blood	60.0	56.6	3.4
Kidneys	92.4	91.2	1.2
Liver	96.8	90.1	6.7
Spleen	93.2	92.2	1.0
Muscle	88.4	88.4	0.0

TABLE X.

Dog Killed by Chloroform 1 Hour after Injection of Phenol.

Organ.	Total phenols per 1,000 gm.	Free phenols per 1,000 gm.	Conjugated phenols.
	mg.	*mg.*	*per cent*
Blood	50.0	33.3	16.7
Kidneys	117.6	100.0	17.6
Liver	117.6	100.0	17.6
Spleen	108.6	92.4	16.2

Baumann found nineteen times more conjugated phenol in the liver than in the blood of a dog poisoned with phenol. One of our dogs succumbed to an overdose of phenol given by stomach and we were able to confirm Baumann's observation. It is probable that in cases of fatal phenol poisoning by stomach the phenol

absorbed from the intestinal tract is temporarily held by the liver and that there is not sufficient time nor proper circulation before the death of the animal for complete distribution to the tissues. Table IX shows that no such concentration of phenols in the liver occurs when the fatal poisoning is caused by injection into the jugular vein.

DISCUSSION.

We may review our conception of phenol metabolism somewhat as follows:

A small part of the tyrosine of the food proteins is broken down by bacterial action into hydroxy-acids such as *p*-oxyphenylpropionic, *p*-oxyphenylacetic, and *p*-oxybenzoic acids, and into volatile phenols, primarily *p*-cresol and phenol. The hydroxy-acids which have no toxic effects are not subjected in any noticeable degree to oxidation or conjugation and are practically completely excreted in the urine in the free state. The volatile phenols which are very toxic even in small amounts are dealt with in an entirely different way. More than half of these toxic phenols are oxidized by the intestinal mucosa, body fluids, and liver parenchyma. The remainder is conjugated in the liver with sulfuric or glucuronic acids. After passing from the liver the conjugated phenols are uniformly distributed to all the tissues and are rapidly eliminated by normal kidneys probably within 12 hours. From the experiments given in the following paper we feel confident that the synthesis of phenol-sulfuric and phenol-glucuronic acids takes place only in the hepatic parenchyma.

SUMMARY.

Intravenous injection of phenols is followed by a prompt and uniform distribution of such substances in the body fluids and tissues.

Following such injection we note a rapid disappearance of free phenols from the blood and a rapid increase in conjugated phenols. This rise in conjugated phenols usually reaches a maximum within 1 hour and thereafter slowly declines with excretion. We note too a uniform distribution of these conjugated phenols throughout the body fluids and tissues.

Ingestion of phenols gives a totally different picture. With a sufficiently large dose some free phenols appear in the blood for a short period—rarely more than 30 minutes. The conjugated phenols show a maximum rise during the first and second hours and subsequent decrease due to renal elimination.

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STUDIES OF LIVER FUNCTION.

III. PHENOL CONJUGATION AS INFLUENCED BY LIVER INJURY AND INSUFFICIENCY.

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In a recent article dealing with the study of liver function Delprat and Whipple (2) review some of the difficulties arising in this work. We refer to that paper for a discussion of the many factors which complicate a critical study of hepatic function, liver injury and repair, together with the great reserve capacity of the liver cells. The ideal liver function test is not at hand and any studies in this difficult field should be of interest alike to the physiologist and the physician. We must keep in mind that any satisfactory *liver function test must include some factor of strain or load* to determine the upper limits as well as the lower levels of liver function.

EXPERIMENTAL OBSERVATIONS.

Tables XI to XVI give the results of some of the experiments we have carried out. The general plan of these experiments was as follows: the dog was first standardized, either after several days of fasting or several days of carbohydrate diet in order to determine the time required by the liver to conjugate the standard dose of *p*-cresol. Liver injury was then produced by one of a number of methods and another test was made to observe the capacity of the injured liver. Tables XI and XIV are given as representative of over a score of standardization experiments on carbohydrate diet or fasting. All of them show surprising uniformity. The percentage conjugation of added phenols and the time required to do this work give a fairly accurate idea of

the ability of the liver to conjugate phenols. In the calculation of the *percentage conjugation* of *added phenols* the assumption is made that the free and total phenols present before ingestion remain constant during the experiment and are not influenced by any of the procedures such as the repeated bleeding or the ingestion of fluid, etc. That this is actually the case is shown in Table XIV where one of three control experiments is reported. These experiments were similar in every way to the other tests performed, except that, instead of the phenol solution, a corresponding amount of water was given by stomach tube.

It may be advisable to explain by example how the *percentage conjugation* of added phenols is calculated:

	Total phenols.	Free phenols.
	mg.	*mg.*
Before ingestion..............................	24	22
After "	28	24

The increase of total phenols after ingestion over total phenols before ingestion is 4 mg. The increase of free phenols after ingestion over free phenols before ingestion is 2 mg. The difference between the two, 2 mg. (or 50 per cent) of the added phenols, has been conjugated. The percentage conjugation of added phenols at any given time after ingestion may, therefore, be calculated by the following simple algebraic formula.

$$\frac{a-b}{c-d}.100 = \text{percentage conjugation}$$

where a represents free phenols after ingestion, b free phenols before ingestion, c total phenols after ingestion, d total phenols before ingestion. In the case above

$$\frac{24-22}{28-24}.100 = 50 \text{ per cent}$$

We have used the method of Davis and Whipple (1) to produce standard liver injury and necrosis with consequent impairment of function. All operative procedures were done under surgical ether anesthesia.

TABLE XI.

Chloroform Injury and Eck Fistula Experiments.

Dog 21-100. White bull, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Mar. 25. Standardization on carbohydrate diet.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	23.1	22.2	3.9	
After "				
10 minutes	26.7	24.0	10.0	50.0
20 "	29.4	23.8	19.0	75.0
30 "	29.4	22.5	27.0	95.0
1 hour	33.3	22.2	33.3	100.0
2 hours	30.0	21.1	29.7	
Apr. 16. Chloroform anesthesia (1½ hrs.). Apr. 18, *p*-cresol ingestion.				
Before ingestion	49.3	48.1	2.4	
After "				
10 minutes	53.5	52.1	2.6	4.8
20 "	54.9	50.5	8.0	57.0
30 "	55.6	50.5	9.2	62.0
1 hour	54.1	47.6	12.0	100.0
2 hours	59.4	54.1	8.9	
Apr. 21. Eck fistula operation. Apr. 27, *p*-cresol ingestion.				
Before ingestion	21.1	20.7	1.4	
After "				
10 minutes	25.0	24.4	2.4	5.1
20 "	28.4	26.7	6.0	17.8
30 "	30.0	27.0	10.0	29.2
1 hour	30.7	26.7	13.2	37.4
2½ hours	30.0	24.7	17.7	55.0
5½ "	25.1	22.4	10.8	57.5
May 13. *p*-Cresol ingestion, conjugation much decreased.				
Before ingestion	35.0	34.0	2.8	
After "				
10 minutes	37.8	36.2	4.2	21.4
20 "	39.4	37.0	6.1	31.8
30 "	39.4	35.7	9.4	61.3
1 hour	42.0	40.0	5.0	14.3
2 hours	41.0	39.0	4.9	16.6

TABLE XI—*Concluded.*

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
May 20. Ligation of hepatic artery. May 24, *p*-cresol ingestion.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	27.0	25.0	7.4	
After "				
15 minutes	35.7	33.7	5.6	0.0
30 "	41.5	39.0	6.0	3.4
1 hour	45.4	42.6	6.2	4.3
2 hours	47.6	45.2	5.0	2.0
4½ "	40.8	36.7	10.0	15.2

May 25. Death. Hepatic insufficiency

Experimental Protocol of Dog 21–100 (See Table XI).

Mar. 25, 33 lbs., healthy. Standardized with 1,650 mg. of *p*-cresol after 4 days carbohydrate diet. No reaction. Apr. 16, 75 minutes chloroform after 4 days fasting. Apr. 18, 35 lbs., 1,750 mg. of *p*-cresol, no reaction. Conjugation about one-half normal. Apr. 21, Eck fistula operation. External surface of liver shows that the chloroform injury has not been completely repaired—the centers of the lobules are hyperemic and stand out distinctly from the opaque periphery. Apr. 27, 30 lbs., 1,500 mg. of *p*-cresol. ½ hour after ingestion the dog is severely intoxicated. Conjugation about one-third normal. Operative wound is partly open and infected. May 1, wound sewed up. May 3, wound again open and sewed up. May 13, slight muscle tremors—prominent distension of abdominal cutaneous veins. 32 lbs., 1,600 mg. of *p*-cresol. No reaction, but very weak. Conjugation about one-third normal. May 20, no tremors, no ascites, collateral circulation less prominent than on May 13. 30 lbs. Arch of hepatic artery tied in two places at 11 a.m. 1,500 mg. of *p*-cresol by stomach tube immediately after operation. Very severe reaction which may, in part, have been due to the ether. Temperature at 1 p.m. 36.2°; at 2 p.m. 37.2°; at 3 p.m. 37.9°. Conjugation one-fourth normal. May 24, very weak and slight muscle tremors. Hematocrit red cells 38 plus. 25 lbs. 1,250 mg. of *p*-cresol, severe reaction. Conjugation less than one-fourth normal. May 25, died 5 days after ligation of hepatic artery.

Autopsy.—May 25. Thorax, heart, and lungs normal. Spleen fibrous and rather pale. Serous surfaces clean except plastic adhesions and yellowish fibrinous exudate about site of hepatic artery operation. Stomach and intestines not abnormal. Kidney and pancreas negative. Hepatic artery ligated in two places and completely occluded.

Eck fistula about 6 mm. long and clean. There must have been considerable flow through this opening or it would have closed. The ligature

above the Eck fistula on the portal vein did not completely occlude the lumen. Lumen probably about 1 mm. in diameter. The knot must have slipped before the second tie was made.

Liver is atrophic but not exactly like the usual Eck fistula specimen. It is not as translucent. The lobules are small and brown at the margins—yellow in the centers, probably necrotic. Some areas are swollen, yellow, and opaque. The lobules here are larger and yellow—necrosis probable. These areas are not numerous, but pretty sizable, 2 × 2 × 5 or 6 cm. for the largest—the volume is estimated as about one-tenth or less of the liver parenchyma.

Microscopic Examination.—Spleen, much pigment and phagocytes. Liver, some sections show extensive hyaline necrosis, involving the centers of the lobules up to 90 per cent in extent—few liver cells escape at the margins of the lobules. Other sections show central atrophy alone (usual Eck picture) with phagocytes full of lipochrome pigment. Others show some evidence of repair, enlarged liver cells, mitoses, etc. Other organs of no importance.

Most instructive are experiments performed on two Eck fistula dogs.

Standardized on Mar. 25, Dog 21-100 showed 95 per cent conjugation within 30 minutes (Table XI). On Apr. 16 a liver injury of about 60 or 70 per cent was produced by chloroform. Table XI, experiment on Apr. 18, shows slightly less than 50 per cent reduction of function. On Apr. 21 the Eck fistula operation was performed. On Apr. 27 the test with *p*-cresol revealed a one-third normal conjugation. A repetition of this test on May 13 shows a slight improvement in function. The arch of the hepatic artery was ligated in two places on May 20 and on testing with *p*-cresol we found that there remained less than 3 per cent of the original capacity of the liver to conjugate phenols. Death of the dog was due to liver insufficiency.

The story of the other Eck fistula dog is similar.

Table XII shows that Dog 21-105 exhibited perfectly normal conjugation when standardized on a carbohydrate diet. On Apr. 5 we performed an Eck fistula operation and a conjugation test on Apr. 12. It appears that only 27.8 per cent of the added phenols had been conjugated 2 hours after ingestion, whereas before the operation 100 per cent conjugation occurred in 20 minutes—the function of the liver on Apr. 12 we may estimate as about 10 per cent of normal. On Apr. 20 another test was made and some improvement noted. The conjugation at this time amounted to about 25 per cent of normal. On May 16 we ligated the hepatic artery in one place, leaving some of the collaterals to the liver patent. It will be seen that the liver function after this operation amounted to somewhat more than 5 per cent of normal.

TABLE XII.

Eck Fistula Experiments.

Dog 21-105. Black, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Mar. 31. Standardization on carbohydrate diet.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion..............	24.3	23.4	3.7	
After "				
10 minutes..................	26.0	23.5	9.6	94.0
20 "	28.2	22.0	22.0	100.0
30 "	29.0	22.5	22.4	100.0
1 hour	35.0	21.8	37.7	100.0
2 hours	33.0	23.0	30.0	100.0
Apr. 5. Eck fistula operation. Apr. 12, *p*-cresol ingestion.				
Before ingestion..............	25.5	24.7	3.1	
After "				
10 minutes..................	33.1	31.2	5.7	14.4
20 "	34.2	32.2	5.8	13.8
30 "	36.8	34.5	6.2	13.2
1 hour......................	37.7	34.5	8.5	19.6
2 hours.....................	36.3	32.5	10.4	27.8
Apr. 20. 15 days after Eck fistula operation.				
Before ingestion..............	27.0	26.7	1.1	
After "				
10 minutes..................	38.0	36.2	4.7	13.6
20 "	38.0	35.1	7.6	23.6
30 "	42.5	38.5	9.4	23.9
1 hour......................	42.0	35.7	15.0	40.0
2 hours.....................	37.0	30.0	18.9	67.0
3 "	33.5	25.0	25.3	100.0
May. 16. Immediately after partial ligation of hepatic artery. Conjugation greatly decreased.				
Before ingestion..............	26.7	26.0	2.6	
After "				
15 minutes..................	43.5	41.7	4.1	6.5
30 "	47.0	44.7	4.8	7.8
1 hour......................	52.5	48.1	8.3	14.3
1½ hours....................	52.0	46.9	9.8	17.3
2½ "	40.9	35.0	14.4	36.6
5 "	37.0	30.2	18.3	59.2
May 18. Death. Hepatic insufficiency and peritonitis.				

Experimental Protocol of Dog 21–105 (See Table XII).

Mar. 27, carbohydrate diet begun. Mar. 31, 29 lbs., slight distemper. Standardized with 1,450 mg. of *p*-cresol. No reaction. Apr. 5, Eck fistula operation. No food. Apr. 6, water and bread. Apr. 7, regulation diet: carbohydrates, vegetables, kaolin, and bones. No meat or milk. Apr. 12, 26 lbs., slight distemper. Moderate ascites. 1,300 mg. of *p*-cresol by stomach tube. Moderately severe reaction. ½ hour after ingestion dog falls down occasionally when trying to walk. 1 hour after dog does not fall down but is still very shaky. Phenol conjugation is about one-tenth normal. Apr. 20, 30 lbs., no distemper. Severe ascites. Collateral circulation on abdominal wall is prominent. 1,500 mg. of *p*-cresol by stomach tube. Moderate reaction. A half hour after ingestion the dog sways from side to side when trying to walk but does not fall. Phenol conjugation about one-fifth normal (a little better than on Apr. 12). May 16, 26 lbs., moderate ascites. Ligature on hepatic artery in only one place, 1,300 mg. of *p*-cresol. Moderate reaction. Dog has apparently recovered late in afternoon. Phenol conjugation less than one-tenth normal. Hematocrit red cell 42 per cent. May 18, dog died 32 to 38 hours after last operation.

Autopsy.—May 18. Ascitic fluid less than at operation—now about 100 cc. The fluid is turbid and the serous surfaces are injected—there is a perforation in the first third of the duodenum and shortly before death there evidently had been an escape of intestinal contents with recent peritonitis. Thorax, heart, and lungs negative. Blood clots normal. Spleen small and fibrous; not much blood. Adhesions about the site of the first operation are numerous and bled easily at the second operation. Liver small, decidedly yellow, due to fat. Lobules show much injury (fat) and perhaps necrosis—there is no edema and the veins are clear. Fistula is about 5 mm. in length and is less than one-half its original length. The edges are smooth and there is no thrombosis. The passage of blood through it was obviously difficult and caused the portal stasis, development of collaterals, and ascites. Intestinal tract negative in general. Duodenum shows a sharp punched out area about 3 × 1 cm. due probably to ligature of the hepatic artery. Kidneys negative.

Microscopic Examination.—Much fat in liver cells (large and small droplets), central necrosis is abundant—about one-third of cell lobules. Kidneys negative. Death explained by liver injury plus terminal duodenal perforation.

Chloroform poisoning gives considerable information as to the conjugation of phenols in the liver. It also points out the fact that *this method is inadequate to measure the high limits of liver capacity.* For example, given a chloroform injury of approximately one-third of the liver parenchyma we may expect a normal or almost normal conjugation of phenols after administration of

the unit dose with the usual routine technique. This means that our test does not reveal the maximum capacity of the normal liver in conjugation of phenols. When we have a chloroform liver injury of one-half to two-thirds of the liver lobule we can then demonstrate a considerable impairment of liver conjugation (compare Table XI, Experiment of April 18). With more advanced liver injury we note a great drop in liver conjugation (compare Table XIV, Experiment of April 22). Finally with a *lethal chloroform injury* involving the greater part (90 per cent or more) of each liver lobule (Table XIII, Dog 21–113) we observe *zero liver conjugation.*

Phosphorus injury gives a similar picture of impaired liver function (Table XIII). It is more difficult to estimate the amount of liver injury in phosphorus poisoning as cell necrosis is not a conspicuous feature of the injury. We note two experiments in Table XIII to show liver function impairment. The injury was fatal in one experiment and very severe in the second animal, yet there was a certain amount of phenol conjugation in both experiments.

We observed no impairment of liver function as shown by phenol conjugation in bile fistula dogs. It is interesting to recall that one liver function test (phenoltetrachlorphthalein) shows a distinct fall in output in bile fistula dogs indicating that the chronic cholangitis which is usually present in such animals interferes with the elimination of phenoltetrachlorphthalein but not with the conjugation of phenols.

Another important control is given by dogs sick with *distemper,* as it might be argued that intoxication of any sort might disturb this conjugation of phenols. One animal (Table XVI) was profoundly prostrated with acute distemper and died shortly after the completion of the experiment yet a normal conjugation followed the administration of the unit dose of *p*-cresol. Autopsy showed the familiar lesions of acute distemper and nothing else. Under these conditions therefore the capacity of the liver to conjugate phenols is in nowise impaired.

From an examination of Table XI, Experiment of April 18, it will be seen that following chloroform injury the initial concentration of total phenolic substances in the blood is more than twice, and, in some cases (fatal chloroform injury, Table XIII),

TABLE XIII.

Chloroform and Phosphorus Injury of Liver.

Dog 21-113. Mongrel, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
May 2. Chloroform anesthesia, 1½ hours. Liver necrosis almost total. Conjugation zero.				
May 3. *p*-Cresol by ingestion.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	103.0	99.2	3.0	
After "				
10 minutes	111.0	107.0	3.6	2.5
20 "	121.5	117.0	3.7	3.8
30 "	124.0	121.0	2.4	0.0
1 hour	135.0	131.0	3.0	0.6
2 hours	133.0	130.0	2.3	0.0
Dog died 2 days after injury. Microscopic examination shows only 1 to 2 rows of surviving liver cells about portal veins.				
Dog 21-112. Black, male. Phosphorus injury, fatal. Conjugation impaired.				
Before ingestion	42.5	42.0	1.2	
After "				
10 minutes	49.5	47.0	5.5	28.5
20 "	51.5	47.6	7.6	37.7
30 "	52.6	46.0	12.5	60.4
1 hour	60.5	50.0	17.3	55.5
2½ hours	58.5	38.5	34.2	100.0
2 mg. of phosphorus per pound body weight, in oil subcutaneously. Test made 1 day after injury. Dog died 36 hours after injection. Autopsy shows fatty degeneration of liver.				
Dog 21-76. Mongrel, male. Phosphorus injury, sublethal but severe. Conjugation impaired.				
Before ingestion	26.7	26.0	2.7	
After "				
10 minutes	32.6	27.0	17.1	83.0
20 "	35.6	29.2	17.7	61.3
30 "	40.8	31.5	22.7	61.0
1 hour	40.0	28.2	29.5	83.5
2 hours	34.2	25.2	26.3	100.0
2 mg. of phosphorus per pound body weight, in oil subcutaneously. Test made 2 days after injury. Dog recovered very slowly.				

four times as high as in the blood of fasting normal dogs or dogs on a carbohydrate diet. We have no definite knowledge regarding all the factors responsible for this pronounced increase, but it is

TABLE XIV.

Chloroform Liver Injury and Controls.

Dog 21-80. Brown, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Apr. 15. Standardization after 4 days fasting.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion...............	30.8	29.4	4.5	
After "				
10 minutes..................	35.2	30.2	14.2	98.0
20 "	37.8	30.2	20.1	89.0
30 "	38.5	28.0	27.3	100.0
1 hour......................	40.0	28.5	28.7	100.0
2 hours.....................	36.4	28.2	22.5	100.0
Apr. 20. Chloroform anesthesia 1 hour, 20 minutes. Apr. 22, *p*-cresol by ingestion.				
Before ingestion...............	31.3	30.0	4.1	
After "				
10 minutes..................	43.5*	41.0	5.7	9.8
20 "	48.5	43.6	10.1	20.9
30 "	47.8	41.8	12.5	28.4
1 hour......................	46.5	40.0	14.0	34.2
2 hours.....................	40.0	33.0	17.5	65.2
Effect of repeated bleedings. Apr. 28. Water given instead of phenol solution.				
Before ingestion...............	27.2	26.8	1.4	
After "				
10 minutes..................	27.0	26.9	0.4	
20 "	26.8	26.8	0.0	
30 "	27.5	27.2	1.0	
1 hour......................	26.9	26.7	0.7	
2 hours.....................	26.5	26.4	0.4	

interesting to speculate about some of them. It has been shown by Dubin (3) that in a fasting dog the concentration of urinary phenols, after an initial drop rises considerably. In our cases

TABLE XV.

Bile Fistula Control.

Bile fistula dog (old white bull, female).

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion...............	22.7	21.5	5.3	
After "				
10 minutes...................	31.2	28.3	9.3	20
30 "	34.5	22.7	34.2	89
1 hour........................	31.1	22.7	27.0	86
2 hours.......................	30.8	20.8	32.4	100

Bile fistula of over 2 years duration—dog normal.

TABLE XVI.

Acute Distemper Intoxication—Control.

Dog 21-98. Black-brown, long haired female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Mar. 23. Standardization.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion...............	28.3	27.8	1.7	
After "				
10 minutes...................	37.0	33.3	10.0	36.5
20 "	43.1	37.4	13.2	34.7
30 "	47.1	40.0	15.0	35.1
1 hour........................	41.7	28.9	30.7	91.7
2 hours.......................	37.7	25.4	32.6	100.0
Apr. 13. Terminal distemper.				
Before ingestion...............	29.0	28 4	2.0	
After "				
10 minutes...................	38.0	33.0	13.1	48.9
20 "	42.4	37.0	12.7	36.0
30 "	45.2	38.3	15.2	38.9
1 hour........................	40.0	29.8	25.5	87.2

Very severe distemper, toxic condition, slight reaction to cresol after 10 minutes, from which dog completely recovered in 30 minutes. Died of distemper after 1 hour. Postmortem: No pneumonia.

of chloroform injury the period of fasting (3 to 4 days) was certainly not long enough to account for the tremendous increase; this is further controlled by the normal fasting dogs (Table XIV and others) in which no such increase occurs. It has been pointed out that the increase of urinary phenols in prolonged fasting is due to a great destruction of proteins. This cell destruction may be one of the factors producing the rise of blood phenols in liver injury. As mentioned before, proteins and their decomposition products such as tyrosine, tryptophane, and other easily oxidizable substances give the blue color with the phenol reagent, and since the increase of conjugated, volatile phenols in these cases is not proportional to the increase in total phenols (the conjugated phenols being practically the same as in normal dogs) the bulk of the additional color-producing bodies may be proteins and their decomposition products other than phenols, or sugars and related substances (Gortner and Holm, 4; Levine, 5).

It was shown in the preceding paper that a large part of the absorbed phenols is destroyed by a process of oxidation entirely different from the synthetic process by which conjugated phenols are produced. It is possible that this oxidative destruction which goes on in most tissues, but to greatest extent in the liver and in the intestinal epithelium, is inhibited or at least greatly reduced by the presence of injured liver tissue, thus adding to the increase in phenolic substances. This seems to be substantiated by the fact that in dogs injured by chloroform the absolute rise in blood phenols after ingestion of *p*-cresol is always greater than in normal dogs. Table XI, Experiment of April 18, and Table XIII, Dog 21–113 show further that with chloroform injury the excretion of ingested phenols may be less rapid than in normal dogs—another less important factor in the increased concentration of phenolic substances in the blood. Delprat and Whipple (2) showed that there is no impairment of *renal* function following a chloroform anesthesia as measured by the elimination of phenolsulfonephthalein.

In accounting for this increased concentration of "phenolic reacting substances" in blood in cases of chloroform injury, we have, in addition to whatever unknown factors may play a part, these possibilities: (1) an increased destruction of body proteins, particularly liver proteins, by chloroform; (2) an inhibition or

lessening of the oxidative destruction of absorbed phenolic substances; and (3) a slowing of the excretion of phenolic substances.

It is interesting to note that this rise in blood "phenols" seldom occurs in dogs whose liver has been injured with phosphorus. Even fatal phosphorus injury shows only a slight increase over the normal level (Table XIII). Nor does this increase occur with bile fistula or Eck fistula dogs. This indicates again the presence of certain "phenol-reacting" substances in the blood of dogs poisoned with chloroform but not necessarily true phenols alone.

DISCUSSION.

The cause of death in Eck fistula dogs has long been a puzzle to physiologists. It has been claimed by many workers that this peculiar intoxication was due to the absorption of the toxic amino-acids as it is well known that heavy meat feeding will precipitate the characteristic intoxication. Unpublished experiments of Van Slyke and Whipple show that there is no abnormal heaping up of amino nitrogen in the blood during periods of meat feeding in Eck fistula dogs. No amino-acids, such as have been noted in severe cases of chloroform poisoning and fatal liver injury, appear in the urine of these Eck fistula dogs. The experiments given in this paper indicate clearly that the Eck fistula liver is incapable of normal conjugation of one toxic radicle, (*p*-cresol). This disability is noted during periods of normal health as indicated by clinically normal reactions. It is at least possible that this impairment of the conjugating powers of the liver is responsible for the toxic developments in the Eck fistula dog. We note that there is no heaping up in the blood of the Eck fistula of any phenol-reacting substances. It will be of considerable interest to study this reaction in the Eck fistula dogs on a high meat diet and during periods of the characteristic Eck fistula intoxication. It is to be recalled that these Eck fistula dogs were maintained on a diet of rice, bread, milk, and bones.

It is significant to note in the tables that severe poisoning with phosphorus will not cause a great rise in the total blood phenols but equally severe poisoning with chloroform will give very high figures for total blood phenols. One suspects that cell necrosis which is so conspicuous in chloroform poisoning is responsible for this difference. This reasoning suggests that a considerable

part of the total phenol of the blood in chloroform poisoning may be due to "*phenol-reacting substances*" quite apart from the phenolic substances.

The method we have used is of interest to investigators but as yet of little practical value to internists. *p*-Cresol is too toxic to be used clinically but further study may enable us to suggest some non-toxic radicle which will test these synthetic activities of the liver parenchyma. It is of some significance that this method is a specific test of liver function, as we have evidence that other body cells are not concerned in the conjugation of phenols.

SUMMARY.

The conjugation of phenols in the body is in nowise disturbed by bleeding periods, by the presence of a bile fistula nor by a lethal intoxication (distemper). Under the conditions of these experiments the reaction to ingestion of a unit dose of *p*-cresol is uniform and associated with a constant amount of phenol conjugation which can be measured quantitatively.

The presence of an Eck fistula modifies this reaction and *reduces* the amount and speed of phenol conjugation. At times the Eck fistula liver function of conjugation may fall to one-third or even to one-tenth of the normal. When the liver circulation is further impaired by partial ligation of the hepatic artery in an Eck fistula dog, we may observe a fall in phenol conjugation to 3 or 5 per cent of normal. Liver exclusion, therefore, will eliminate phenol conjugation.

The presence of a slight liver injury due to chloroform or phosphorus may not modify the phenol conjugation. Extensive liver injury due to these poisons will always lessen phenol conjugation. Extreme and *fatal liver injury* (chloroform) *will reduce phenol conjugation to zero.*

These observations lead us to conclude that *phenol conjugation* is a function of *liver parenchyma cells* and of no other body cells.

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THE EFFECT OF HYDROGEN ION CONCENTRATION UPON THE DETERMINATION OF CALCIUM.

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McCrudden's (1, 2) method has come to be recognized as the best for the accurate determination of calcium. The method is to precipitate the calcium as oxalate in the presence of sufficient ammonium chloride to hold the magnesium oxalate in solution, and of sufficient acid to hold the calcium oxalate partly in solution. Sodium acetate is then added to decrease the acidity and to precipitate the rest of the calcium oxalate on the crystals already formed. Thus, large crystals are made which are easy to filter and are not contaminated with occluded magnesium or calcium phosphate.[1] The amount of acetate is selected so as to give a solution not acid enough to dissolve the calcium oxalate, nor alkaline enough, if cold, to allow calcium phosphate to precipitate. These directions are empiric and must be followed accurately, as the author cautions.[2] Just what determines the amount of acetate is not definite, for he states that in the ash of feces 15 instead of 8 cc. are required.[3]

The sodium acetate added regulates the acidity of the solution. Unfortunately, at the time McCrudden wrote his article, Sörensen's (3), Michaelis' (4), and Clark's (5) monographs on hydrogen ion concentration were not published. In 1913, Hildebrand (6) of the Bureau of Standards showed the necessity of a systematic investigation of analytical methods with regard to the hydrogen ion concentration. Such information is not as yet available for

[1] McCrudden (1), p. 99.
[2] McCrudden (1), p. 100.
[3] McCrudden (2), p. 198.

many types of analysis. Except that of Kramer and Tisdall (7) there is none for calcium. We have, therefore, critically examined McCrudden's method in relation to hydrogen ion concentration.

Theory Underlying the Precipitation of Calcium as Oxalate.

The determination of calcium in the presence of magnesium and phosphate depends primarily upon the solubility product of the various precipitates involved; secondarily, upon the hydrogen ion concentration.[4] As a development of the last 30 years in the chemistry of solutions, the theory of ionization has been applied to the problem of precipitation of nearly insoluble substances with great success.

Solubility Product.— Stieglitz (8) and Noyes (9) have presented very carefully in their manuals of qualitative analysis the relation between ionization and precipitation. The basic principle is the mass law, which can be stated.[5]

$$\frac{(A^+) \times (B^-)}{(A\ B)} = K$$

In non-mathematical terms, when a substance is present in water an equilibrium is established between the concentration of the undissociated salt in solution and the concentration of the ions. The value, K, is a constant depending upon the nature of the particular salt. Or, when there are two ions present which form an insoluble compound, precipitation occurs until the product of the concentration of the ions divided by the concentration of undissociated salt is a constant which is the K for that salt.

A simpler relation which holds with sufficient accuracy for practical purposes is that the product of the ions is a constant. This constant is known as the "solubility product."

$$(A^+) \times (B^-) = K$$

Obviously, we decrease the amount of either ion present by increasing the other ion. If we double A^+ we halve B^-. Hence, adding more A^+ causes more and more complete precipitation

[4] The question of the problem of occlusion and the conditions for obtaining large crystals have been discussed by McCrudden (1), p. 99.

[5] Parentheses about a symbol mean concentration. Thus, H^+ means hydrogen ion; (H^+) means hydrogen ion concentration.

of B^-. Thus, one adds an excess of oxalate ions, so that the calcium is more completely precipitated. The value of the solubility constant is very important for it determines which salt will precipitate. The salt having the smaller solubility product will precipitate and the other will remain in solution. If all the factors are known a quantitative expression can be calculated rigorously from the mass law. The solubility product determines whether calcium will be precipitated as a phosphate or carbonate or oxalate in solutions containing these acids. The solubility products of the various salts to be considered are given in Table I.

Effect of Hydrogen Ion Concentrations.—Acids on dissociation always give hydrogen ions, H^+. The extent of the acidity depends upon the concentration of the hydrogen ions, (H^+) (10). The effect of hydrogen ions in precipitation can best be discussed under three heads: (*a*) the effect on basic salts, (*b*) the change in the ionization constant of the acid radicals, and (*c*) the suppression of ionization of weak acids.

The effect of acid on basic salts is to depress the ionization of the hydroxyl ions; for substances in solution are related to the ionization of water. Water itself gives H^+ ions and also OH^- or basic ions. The relation of the ions is expressed by the mass law:

$$\frac{(H^+) \times (OH^-)}{(H_2O)} = 10^{-14}$$

When one adds H^+ ions to water one depresses the ionization of the hydroxyl ions since the product of these ions is a constant. Thus, in the case of magnesium hydroxide, by adding acid one decreases $(\bar{O}H)$ to such an extent that in acid solution its solubility product is never reached and hence no magnesium hydroxide can precipitate.

By adding acid one changes the ionization constant of the acid. Thus, in the case of the tri-basic phosphate the addition of H^+ ions gives the following reaction:

$$\bar{\bar{\bar{P}}}O_4 + H^+ = H\bar{\bar{P}}O_4 + H^+ = H_2\bar{P}O_4$$

The tri-basic salt can only exist in alkaline solution and as one increases the hydrogen ion concentration the di-basic and monobasic salt must be formed. Thus, in an acid system there is no calcium phosphate but only the more soluble di-basic and mono-

basic calcium phosphates. The solution, if quite acid, about pH 3.0, will have the same effect on calcium oxalate.

$$H^+ + (\bar{C}_2\bar{O}_4) \overset{\longrightarrow}{\rightleftharpoons} (H\bar{C}_2O_4)$$

By adding acid to a solution containing ions of a weak acid one converts the highly ionized salt into a slightly ionized acid, according to the equation:

$$2H^+ + (\bar{C}_2\bar{O}_4) \overset{\longrightarrow}{\rightleftharpoons} H_2C_2O_4$$

Thus one removes oxalate ions from the solution by adding acid. If the ionization is repressed below the solubility product no precipitate is formed.

Each of these factors: the effect on basic salts, the change in the ionization constant of the acid, and the suppression of ionization, influence the precipitation. Therefore, we shall consider each of the salts that may be formed in the course of the analysis of calcium in the presence of magnesium and phosphates, in an acid solution of pH 4.0 to 6.2.

Calcium Oxalate and Magnesium Oxalate.—These salts are precipitated in the presence of an excess of oxalate ions. The question is whether there is any danger of precipitating magnesium with the calcium. This has been discussed in part by Kramer and Tisdall (7). The solubility product of these salts is sufficiently different so that Gooch (11) recommends that even in the presence of ten times the amount of magnesium it is not necessary to carry out double precipitation. In acid solution this difference in solubility product is even more marked.

Acid added to a solution of calcium and magnesium oxalates favors the precipitation of the calcium in two ways. It forms the acid salts which are more soluble. The acid salt of calcium oxalate, at this acidity (pH 4.0 to 6.2) is not formed in sufficient amount to have any appreciable effect on the solubility. No study of the magnesium salts has been made, but from general evidence it is concluded that this would be more readily affected by acids and hence more soluble.

Second, the addition of acid suppresses ionization, in this case from about 40 to about 30 per cent. The diminished ioniza-

tion affects the calcium oxalate much less than the magnesium oxalate as the former has a much smaller solubility product. Any acidity not great enough to form acid calcium oxalate will be a factor in preventing the formation of magnesium oxalate and in favoring the precipitation of calcium oxalate.

Magnesium Hydroxide and Magnesium Ammonium Phosphate.—Both salts are extremely insoluble but since they do not occur in acid solutions they do not precipitate. Hildebrand (6) says that the former precipitates at pH 7.4 to 8.0 and that the latter first appears at pH 6.6.

Mono-, Di-, and Tri-Basic Calcium Phosphate.—The salts of calcium form a very difficult problem since as found by Cameron (12) and his collaborators and many others, these salts vary in their composition according to the source, are decomposed by water, and take months to come to equilibrium.

For orientation we attempted to find the pH of saturated solutions of the mono-, di-, and tri-basic salts of calcium phosphate. These salts (Baker's analyzed) were washed with distilled water. The acidity of the supernatant liquids, determined by the colorimetric method (5), became roughly constant at room temperature in 24 hours and gave the following values:

$$CaH_4(PO_4)_2 = \text{pH } 5.0$$
$$CaHPO_4 = \text{pH } 7.4$$
$$Ca_3(PO_4)_2 = \text{pH } 6.8$$

We found that no calcium solution more acid than pH 4.0 which contained phosphates yielded a precipitate after boiling, but Patten and Mains (13) report a precipitate at pH 2.3 at 26°. But the more acid the point at which these salts precipitate, the greater is the proportion of phosphate to calcium. And, as one can see by glancing at Table I, the more acid the salt the greater is the solubility product. Therefore, at pH 4.0 to 6.4 there is no danger of any calcium being present as phosphates. At this acidity the calcium phosphates have a solubility product more than a million times as great as calcium oxalate. Breazeale (14) remarks it is quite safe to precipitate calcium phosphate quantitatively as calcium oxalate by adding oxalic acid. Therefore, there is no danger of the precipitation of calcium acid

phosphate if the precipitation of calcium oxalate is carried out at the proper acidity.

TABLE I.

*Data on Solubilities.**

Salt.	Per liter.	Mols per liter.	Solubility product.
	gm.		
CaC_2O_4	0.0055	0.000044	1.9×10^{-9}
$Mg(OH)_2$	0.009	0.00015	3.5×10^{-12}
$MgNH_4PO_4$	0.05	0.0068	3.0×10^{-7}
MgC_2O_4	0.302	0.0027	4.8×10^{-6}
$Ca_3(PO_4)_2$	0.01	0.00033	2.8×10^{-18}
$CaHPO_4$	0.2	0.00147	2.0×10^{-8}
$CaH_4(PO_4)_2$	18.0	0.77	4.0×10^{-1}

* The figures are compiled from Landolt-Börnstein, Physikalisch-chemische Tabellen; A. Seidell, The solubilities of inorganic substances; The Chemische Kalender, 1914—Dictionary of Solubilities, etc.

EXPERIMENTAL APPLICATION OF THE THEORY.

It is important to verify, first, the most acid limit of acidity; second, the least acid limit; and third, the best method of obtaining the desired acidity.

Most Acid Limit.—The most acid limit is the point at which calcium oxalate begins to be converted into the more soluble acid calcium oxalate. McCrudden has determined the amount of sodium acetate, which, under the conditions of his procedure will prevent the solution of calcium oxalate.[6] We determined colorimetrically the hydrogen ion concentration of solutions precipitated according to his directions. In all solutions more acid than pH 4.4, which contain less than 6 cc. of sodium acetate, the results are low. Further experiments in Table II show that correct determinations are made at pH 4.0, which is, therefore, the most acid limit for the determination of calcium oxalate.

The Least Acid Limit.—The least acid limit is the point at which magnesium ammonium phosphate and magnesium hydroxide precipitate. According to Hildebrand this is pH 6.6 to 7.6. To fix this point experimentally we carried out the precipitation

[6] McCrudden (1), p. 90.

TABLE II.

*The Amount of Sodium Acetate and the Resulting Acidity.**

No.	20 per cent sodium acetate.	pH calculated.	pH determined.	Calcium.
	cc.			*mg.*
1	0		1.3	30.1
2	4	2.8	2.8	32.0
3	5	4.0	4.0	33.1
4	6	4.4	4.4	33.1
5	10	4.8	4.8	33.3
6	20	5.3	5.2	33.1
7	50	5.7	5.6	33.2
Theory....				33.2

* In solutions more acid than pH 4.0 the results are low; 50 cc. of sodium acetate are not an excess and give correct results.

of calcium at varying acidities and determined the amount by both the gravimetric and volumetric methods. By this procedure it is possible to determine faulty results and also the cause of error. Titration with permanganate determines the oxalates. If phosphates are present and contaminating the calcium oxalate they will not affect the result. By the gravimetric method one determines the calcium as oxide and the oxalates are destroyed. Phosphates will cause the results to be too high. Reasoning thus, one can deduce the following:

Precipitate consisting of:	Method.	
	Gravimetric.	Volumetric.
Calcium oxalate alone..............................	Correct.	Correct.
" " and calcium phosphate............	High.	Low.
" " " magnesium ammonium phosphate..	"	Correct.
Calcium oxalate and magnesium oxalate...........	"	High.
" " " " hydroxide.........	"	Correct.

Therefore, we took samples of calcium chloride. To these we added phosphates and made determinations by both volumetric and gravimetric methods at pH 7.4. Correct results were obtained by both. To a second sample we added magnesium sul-

fate and made determinations by both methods at pH 7.4. Correct results were obtained by both. Then to a third series of samples we added both phosphates and magnesium. The acidity was adjusted by ammonia to pH 5.6 to 7.4. The results are given in Table III.

From these data it is clear that in the precipitation of calcium oxalate, neither magnesium oxalate nor hydroxide give false results if no phosphate is present. Calcium phosphate does not give false results even in slightly alkaline solution when no magnesium is present; but if magnesium is also present, magnesium ammonium phosphate precipitates if the solution is more alkaline than pH 5.6.

TABLE III.

*The Least Acid Limit for Precipitating Calcium Oxalate.**

Substance present.	pH	Gravimetric Ca.	Volumetric Ca.	Ca present.
		mg.	*mg.*	*mg.*
Ca and PO_4..................	7.4	84.9	84.4	
Ca and Mg....................	7.4	84.8	84.4	
Ca, Mg, and PO_4.............	5.6	84.9	84.7	
" " " "	6.0	86.0	84.5	
" " " "	6.6	92.0	84.9	
" " " "	7.0	94.3	84.9	
" " " "	7.4	100.2	84.8	84.6

* The least acid limit for the determination of calcium oxalate by the gravimetric method is pH 5.6. At points less acid magnesium ammonium phosphate is precipitated.

The Best Method of Obtaining the Desired Acidity.—When sodium acetate is added to a mixture of hydrochloric and oxalic acids, the stronger acids form salts with sodium and there remains in the solution acetic acid in the presence of acetates. This is an excellent buffer mixture and regulates the hydrogen ion concentration. If one knows the ratio of the acetic acid to the sodium acetate, with Walpole's (15) chart, one can estimate the pH. This is given in Table II under the column "pH calculated." How closely this approximates the pH as actually determined is shown by comparing these values with those given in the same table under the column "pH determined." As the ratio of acetic acid to sodium acetate becomes smaller the pH approaches 7.0 as a limit.

McCrudden's fear that calcium phosphate would precipitate unless there were between 6 and 10 cc. of sodium acetate is groundless. To test this point experimentally we made up a solution containing 2 cc. of 10 per cent calcium chloride, 2 cc. of 10 per cent magnesium sulfate, and 2 cc. of 10 per cent sodium acid phosphate. These we precipitated according to McCrudden's directions: 10 cc. of 0.5 N hydrochloric acid, 10 cc. of 25 per cent oxalic acid, 10 cc. of 3 per cent ammonium oxalate, cooled and to them added varying amounts of 20 per cent sodium acetate solution. The results are shown in Table II. Thus, even 50 cc. of acetate are not an excess and give correct results.

We found, by experiment, that it makes no difference whether one regulates the acidity by acetic acid and sodium acetate mixtures or more conveniently and quite as accurately by adding ammonia to the acid phosphates present. We have, therefore, developed a method based upon this conclusion, the details of which are given in the following paper.

Limits of Acidity.—From the above discussion it is clear that the limits of acidity for the precipitation of calcium by oxalic acid in the presence of magnesium and phosphates are determined. The most acid limit is pH 4.0. If the solution is more acid than this the calcium oxalate is partly converted into acid calcium oxalate which is so soluble that it is not quantitatively precipitated. The least acid limit is pH 5.6. If the solution is less acid appreciable amounts of magnesium ammonium phosphate precipitate and contaminate the calcium oxalate.

SUMMARY.

The hydrogen ion concentration is an important factor in making a calcium determination. If the solution is more acid than pH 4.0 calcium oxalate is dissolved. If the solution is less acid than pH 5.6, magnesium ammonium phosphate is precipitated.

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A RAPID AND ACCURATE METHOD FOR CALCIUM IN URINE.

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McCrudden's method (1, 2) for the determination of calcium in urine, at present probably the most accurate and satisfactory of all the calcium methods, is open to the serious criticism that it requires platinum dishes, and is laborious and time-consuming. Not infrequently it takes 24 hours to filter the urine and wash the precipitate. After filtration the ashing and weighing usually occupy an additional day, sometimes longer. McCrudden endeavored to shorten the method by titrating the precipitated calcium oxalate with potassium permanganate, but as the precipitate was contaminated with uric acid obtained too high readings. He states:[1]

"I have concluded, therefore, if calcium oxalate, precipitated from urine, is contaminated with uric acid, as indicated by the reddish color of the precipitate, the calcium should not be determined by titration."

It is very questionable whether color is a good indication of the presence or amount of uric acid, since pure uric acid is colorless. The urinary uric acid, as is well known, depends for its coloration upon the presence of urinary pigments. Because of the above warning, and our experience, we have always relied on McCrudden's method only after ashing and weighing the calcium as calcium oxide. We felt that his method would be improved if we could avoid gravimetric analysis. Such a rapid clinical method for the determination of calcium in the urine is, therefore, desirable.

The method about to be presented, has the advantage of increasing the speed of the determination without appreciably

[1] McCrudden (2), p. 194.

decreasing the accuracy. The problem before us was to precipitate the calcium as calcium oxalate avoiding contamination with uric acid, and then to titrate the precipitate with potassium permanganate.

We first attempted to remove the uric acid by filtration after adding sufficient acid to convert the urates to uric acid and also to dissolve any calcium oxalate present. This is inadequate since considerable time is required and uric acid is very slowly and not quantitatively precipitated from urine. We also object to filtration, which McCrudden recommends for an initial procedure without adding acid, on the ground that any calcium combined with oxalic acid would be held back by the filter paper and lost.

We then searched for some solvent of uric acid with which the precipitate of calcium oxalate and uric acid might be washed on the filter or which would keep the uric acid in solution. Various solvents were tried. Hot sodium carbonate readily dissolved the uric acid but caused the readings to be much too low. Hexamethyleneamine was found to be very inefficient. Piperidine, a noted uric acid solvent, rendered the urine highly alkaline, dissolved the uric acid by virtue of its alkalinity, and was therefore inapplicable. No satisfactory solvent was found.

To eliminate the uric acid, the urine could be either dry ashed or ashed in the wet way following the Neumann method (3). Both these methods have been utilized in determining calcium. There is no objection to them except for the time and labor involved. We thought we might save time and avoid technical difficulties; and if we could destroy the uric acid by oxidation, we would accomplish in a short time, without evaporating the urine, the same purpose as ashing. Any substance which would reduce permanganate would be removed by oxidation.

We tried concentrated solutions of potassium permanganate on theoretical solutions containing calcium and uric acid with correct results. With urine, however, a surprising amount of this reagent was needed for complete oxidation. And this left large quantities of manganous ions, which, in the presence of oxalate ions, were difficult to hold in solution. It is not impossible that this method may be applicable, since manganous oxalate, like magnesium, is held in solution by ammonium chloride (4). We sometimes obtained an impure precipitate.

We then used ammonium persulfate and obtained excellent results (5). This reagent has the advantage of being relatively cheap, of being a strong uric acid-oxidizing agent, and an inefficient oxalate-oxidizing agent. In fact on theoretical grounds it is practically ideal for the purpose. When oxidation is complete, instead of manganous ions, we have sulfate and sulfite ions in solution, and these are not objectionable.

Ammonium persulfate is a white crystalline, deliquescent substance, which deteriorates on exposure to the air. When first put into solution it crackles with the evolution of a gas that smells like ozone. When added to the urine it froths. These properties we have taken as a rough index of its activity. A sample we obtained from Eimer and Amend was very satisfactory, but contained an appreciable amount of calcium. A blank should be run, therefore, for the calcium content. The reagent oxidizes best in an acid medium.

After the urine has been oxidized the calcium is precipitated as oxalate. The method as suggested by McCrudden may be followed.[2] He uses sodium acetate to regulate the acidity. We carry out the precipitation between definite limits of hydrogen ion concentration (6). We have found that the optimum lies between pH 4.8 to 5.2. Therefore, we use 1 drop of methyl red as an indicator. If the color fades rapidly we add a second drop of indicator solution. This indicator almost exactly covers the desired range of pH. It gives a red color when the acidity is as great or greater than pH 4.6, a yellow color when the acidity is less than pH 6.0, and an intermediate color between pH 4.8 to 5.4. This is the color desired. When one uses methyl red as an indicator one may use sodium acetate to decrease the acidity but it is not necessary. We use NH_4OH and bring the solution to the proper end-point, as shown by the color of the indicator. The important condition is to have the solution at the proper hydrogen ion concentration.

When the calcium has been precipitated as described above it is allowed to stand over night. (McCrudden shakes the mixture 10 minutes instead of allowing it to stand.) It is then filtered. Halverson and Schulz (7) have suggested filtering through a

[2] McCrudden (2), p. 199.

Gooch crucible made with specially prepared and ignited asbestos. This is unnecessary; for if the urine has been oxidized with persulfate it filters almost as readily as water. A good grade of hardened filter paper is easier to use. When several determinations are made at once it is practically as rapid as preparing the Gooch crucible and there is no chance of the filter's not retaining the precipitate. The precipitate is then washed back into the original flask and titrated with permanganate.

Reagents required.	
Ammonium persulfate.	Nitric acid (sp. gr. 1.42).
Oxalic acid, 2.5 per cent.	Sulfuric acid (sp. gr. 1.84).
Methyl red, 0.02 per cent in 50 per cent alcohol.	Ammonium hydroxide (sp. gr. 0.9).
Potassium permanganate, 0.05 N.	Sodium oxalate, Sörensen 0.05 N.

Details of the Method.

1. To 100 cc. of unfiltered urine in a 250 cc. Erlenmeyer flask add 5 cc. of concentrated HNO_3 or H_2SO_4, and one spoonful containing 3.0 to 4.0 gm. of ammonium persulfate. Insert a funnel in the flask to prevent spattering.

2. Boil and keep near the boiling point on a hot plate, or over a low flame, for 1 hour, or until reduction of the ammonium persulfate is complete, as evidenced by an absence of frothing when the flask is agitated. The solution at this point is pale green in color.

3. Add 10 cc. of 2.5 per cent oxalic acid.

4. Cool to room temperature.

5. Neutralize with ammonium hydroxide, using 1 drop of methyl red, as an indicator.

6. Cool to room temperature.

7. If the color is now red, the solution may be brought to the desired color by a few drops of ammonium hydroxide. pH 4.8 to 5.2.

8. Let stand over night (McCrudden shakes the mixture for 10 minutes instead of allowing it to stand).

9. Filter. Whatman No. 50 hardened filter paper, 12.5 cm., has been found satisfactory. Wash the precipitate and flask three times with distilled water, filling the filter two-thirds full each

time and allowing to drain. Break a hole in the filter paper, and wash back the precipitate into the original flask, first with distilled water, and then with hot dilute sulfuric acid, bringing the volume to about 100 cc.

10. Add 10 cc. of concentrated sulfuric acid, and heat to 70–80°C.

11. Titrate with 0.05 N potassium permanganate, taking as an end-point the first color that persists 15 to 30 seconds. If, as occasionally happens, the precipitate is colored red by the methyl red, and so colors the solution to be titrated with permanganate, this color does not interfere with the end-point, as it is quickly oxidized. The indicator is not present in sufficient amount to cause any appreciable difference in the titration. The usual precautions should be taken of standardizing the permanganate each day with standardized oxalate. 1 cc. of 0.05 N $KMnO_4$ = 0.001 gm. or 1 mg. of calcium.

EXPERIMENTAL RESULTS.

In the experiments here given no effort was made to obtain the highest degree of accuracy; they represent that obtainable in any physiological laboratory by an analyst of moderate experience. In any series of determinations reported no results have been omitted.

Our standard solutions of calcium were made from calcite, and checked by McCrudden's gravimetric method. A solution of $CaCl_2$, which contained 0.0830 gm. of Ca per 25 cc., was checked by McCrudden's method and gave the following results: 0.0831, 0.0832, 0.0831.

The results on theoretical solutions of calcium are given in Table I. A sample of urine, to which uric acid was added, analyzed by McCrudden's gravimetric, McCrudden's volumetric, and the authors' methods, yielded the results shown in Table II. On another sample of urine the calcium content was determined by the authors' method. To portions of it were added known amounts of calcium. The results are given in Table III. Six determinations were then made on a sample of urine by the authors' method and six determinations on the same urine by McCrudden's gravimetric method. One of these latter was unfortunately lost. The results are given in Table IV.

TABLE I.

Theoretical Solutions by Authors' Method.

Calcium taken.	Uric acid.	Ammonium persulfate.	Calcium present.	Calcium found.
gm.	*gm.*	*gm.*	*gm.*	*gm.*
0.0000	0.0	4.0	?	0.0002
0.0332	0.0	4.0	0.0334	0.0337 0.0332
0.0332	0.2	4.0	0.0334	0.0334 0.0339

TABLE II.

Urine Plus Uric Acid.

Urine.	Uric acid.	Method.	Calcium.
cc.	*gm.*		*gm.*
100	0.2	McCrudden's gravimetric.	0.0342 0.0345 0.0345
100	0.2	McCrudden's volumetric.	0.0541 0.0472
100	0.2	Authors'.	0.0344 0.0342 0.0346

TABLE III.

Recovery of Added Calcium by Authors' Method.

Urine.	Calcium added.	Calcium obtained.	Added calcium recovered.
cc.	*gm.*	*gm.*	*gm.*
100	0.0000	0.0113 0.01165 0.0116	
100	0.0165	0.0272 0.0284 0.0274	0.0162

TABLE IV.

Calcium in Urine.

Urine.	McCrudden's gravimetric method.	Authors' method.
cc.	*gm.*	*gm.*
100	0.0152	0.0160
	0.0168	0.0159
	0.0176	0.0161
	0 0158	0 0158
	0.0148	0.0161
	Lost.	0.0159
Average.............	0.01604	0.01596

DISCUSSION.

The method yields correct results with theoretical solutions containing calcium and uric acid. The calcium in urine can also be determined accurately and added calcium is recovered. Added uric acid does not affect the results by our method but gives high results if not oxidized. The advantages of the method described are: that by oxidation of the urine the resulting solution filters nearly as rapidly as water; the conditions for the precipitation are put on a rational basis by bringing the solution to the proper hydrogen ion concentration, instead of adjusting the acidity empirically with sodium acetate; the removal of the uric acid by oxidation permits the rapid and convenient method of titrating the calcium oxalate with potassium permanganate, instead of conversion to calcium oxide and weighing; and it permits rapid and accurate determinations of calcium in urine without requiring platinum. The method is accurate within 1 per cent in determining 30 mg. of calcium.

CONCLUSION.

1. Calcium in the urine can be accurately determined, if the urine is oxidized with ammonium persulfate.

2. The calcium is precipitated as the oxalate at pH 4.8 to 5.2, and titrated with 0.05 N potassium permanganate.

3. The method requires less than one quarter the time necessary for gravimetric determinations.

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REASONS FOR BELIEVING THAT RESPIRATORY X IS NOT C_H.

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Low oxygen stimulates respiration. So also does CO_2, but through the C_H of the blood. Following the principle of economy of causes most investigators have held that low oxygen must also alter the C_H of the blood or of the cells of the respiratory center itself. Against this view is the fact that low oxygen induces not acidosis but alkalosis; also the fact that CO_2 stimulates respiration even after vagus section, but low oxygen apparently does not. Under morphine the responsiveness to CO_2 is greatly reduced, while low oxygen is still an effective stimulant.

Respiratory *X* is the hypothetical substance occurring in the blood under low oxygen. It is not an acid. Some evidence suggests that it does not act upon the respiratory center but stimulates the pulmonary vagal endings. It might be a sulfur compound, for H_2S is a powerful respiratory stimulant capable of causing overbreathing followed by fatal apnea. After vagus section sulfide is merely depressant; its stimulant action is, therefore, like that which respiratory *X*, by hypothesis, should have upon the pulmonary endings. Sulfide is rapidly oxidized in the blood. It does not act through C_H. Recent, and as yet, unpublished investigations, indicate that low oxygen must act through a substance of these physiological properties, although apparently respiratory *X* cannot be simply H_2S.

The following experiments were mentioned: On Pike's Peak merely squeezing a hand bulb or even opening and closing the fist sufficiently rapidly to fatigue the forearm caused, in Y. H. and some others, but not in all persons, marked hyperpnea. At sea level a similar experiment can be performed, but only on very sensitive subjects, by sawing wood, using both arms vigorously, but no other muscles. The significant fact is that the hyperpnea that is induced is followed by apnea. Apparently some substance, in addition to CO_2, is produced in the overworked and

thus in anoxyemic muscles, which passing into the blood causes overbreathing. It is quite certain that the condition induced in the blood by overbreathing is alkalosis (low ratio of H_2CO_3: $NaHCO_3$), not acidosis. Therefore, the substance is not an acid, but a respiratory stimulant of some other type.

CARBON DIOXIDE AS AN INHIBITANT OF CELL GROWTH.

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Comparative experiments carried out on dividing sea urchins' eggs indicate that carbon dioxide exerts a vastly greater inhibitory action than mineral acids, and that the effect of carbonic acid bears no relation to the hydrogen ion concentration. In a single series of experiments carried out under comparable conditions, carbon dioxide exerted an inhibitory effect at pH 8, and completely prevented cell division at pH 6.3, while hydrochloric acid exerted little or no inhibitory effect at pH 5.8 and development was still found at pH 4.6.

A similar difference is observed in studying the inhibitory effect of acids on the maturation of the starfish egg. It is suggested that this specific influence of carbon dioxide is attributable to its greater solubility in organo substances than in the aqueous phase and the experiments indicate that carbonic acid may play an extremely important rôle as a specific regulatory substance in cell growth.

THE ERYTHROPOIETIC ACTION OF GERMANIUM DIOXIDE.

By F. S. HAMMETT and J. E. NOWREY, Jr.

(*From The Wistar Institute of Anatomy and Biology, Philadelphia.*)

The subcutaneous injection of germanium dioxide solutions in adult male or female albino rats produces a marked erythrocythemia. There is no evidence of any toxic action of this compound, even when given in doses up to 180 mg. per kilo of body weight. The erythrocythemia persists for at least 5 weeks after the last dose when large doses are given. It persists for at least

2 weeks after the administration of relatively small doses. There is no accompanying leucemia.

Histological preparations of the liver, spleen, and bone marrow show the following effects have been produced. There is in most cases a dilatation of the hepatic capillaries and a relative engorgement of these with erythrocytes. There is no evidence of the taking on by the liver of its pristine erythropoietic function. The impression is given by the sections cut from the spleens that here too there is an increased number of erythrocytes which is accompanied by a more dense accumulation of cells in the Malpighian corpuscles. This, however, is merely an impression and not of significant magnitude to be validly distinctive. There is neither evidence for an increased red cell destruction by the splenic phagocytes nor evidence that new red cell formation is taking place in this organ. In the bone marrow there is ample evidence that this tissue has been stimulated to the formation of a larger number of nucleated erythrocytes than is taking place in the bone marrow of the control animals. This is verified by counts made of these types of cells.

An examination of the smears made for the determination of the differential counts of the white blood cells showed an entire absence of nucleated red cells. Nevertheless, it was found that the smears from those rats which had received the germanium injections contained more young red cells per unit area than did the smears of the controls. The index of the young red cells was the presence of polychromatic staining erythrocytes.

On the basis of these findings it is concluded that germanium dioxide is an erythropoietic agent of remarkable potency and that the source of the erythrocythemia produced by it is not a liberation of cells from some deposit within the organism, but is a formation of new cells by the bone marrow which has been stimulated to increased activity by the compound used.

IS THERE A SUBSTANCE OTHER THAN FAT-SOLUBLE A ASSOCIATED WITH CERTAIN FATS WHICH PLAYS AN IMPORTANT RÔLE IN BONE DEVELOPMENT?

By E. V. McCOLLUM and NINA SIMMONDS,

(From the Laboratory of the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

and P. G. SHIPLEY and E. A. PARK.

(From the Departments of Pediatrics, the Johns Hopkins University, Baltimore, and School of Medicine, Yale University, New Haven.)

A series of experiments has been conducted which was designed to show the comparative values of butter fat and of cod liver oil in protecting young rats against the injurious effect of a pronounced deprivation of calcium. The basal diets contained about one-fifteenth the optimal amount of calcium and nearly the optimum of phosphorus.

Cod liver oil exerts a very marked protective influence, enabling the animals to grow, be fairly fertile, and appear well nourished for a considerable period under dietary conditions where without it they fail to grow at all and die early. We were unable to demonstrate much difference in the degree of protection afforded by 1 and 3 per cent of the oil.

With the same diets containing butter fat instead of cod liver oil very little protection was afforded even when 3, 10, and 20 per cent of fresh butter fat was supplied.

When in a series of experiments the calcium content of the diet was increased by small increments from 0.045 to 0.245 per cent through the addition of calcium carbonate, the differences between the nutritive effects of cod liver oil and of butter fat tend to disappear and do entirely disappear as the calcium content approaches the optimal, which is about 0.641 per cent.

The results suggest that either (*a*) cod liver oil contains in abundance a dietary essential which is contained but sparingly in butter fat, or (*b*) that at least two organic nutritive principles are associated with certain fats, and that both butter fat and cod liver oil contain each, but in proportions widely different.

THE EFFECT OF DIETS VERY HIGH IN PHOSPHORUS AND VERY LOW IN CALCIUM ON THE DEVELOPMENT OF THE BONES IN YOUNG RATS.

By E. A. PARK and P. G. SHIPLEY,

(*From the Departments of Pediatrics, the Johns Hopkins University, Baltimore, and School of Medicine, Yale University, New Haven.*)

and E. V. McCOLLUM and NINA SIMMONDS.

(*From the Laboratory of the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.*)

Rats fed diets low in calcium but high in phosphorus grow slowly and remain stunted. They seem nervous, reacting in an exaggerated manner to slight stimuli. One or two of them died suddenly when handled. The behavior taken in conjunction with the composition of the diet naturally suggested the possibility of tetany.

The bones were thin. The enlargements of the costochondral junctions and ends of the long bones of the limbs were slight or lacking. Fractures were not found.

Microscopically, the bones showed a fairly constant condition. The cartilage was in most instances calcified. The trabeculæ were thin and exceedingly numerous both at the epiphyseal end of the shaft and in the cortex. The trabeculæ were bordered with not very thick zones of osteoid. A thin fibrous tissue invested the trabeculæ, and when it filled in the spaces between the trabeculæ, it gave rise to pictures corresponding to the "fibrous marrow" so commonly seen in rickets. Scattered around the trabeculæ were many mononuclear cells with basophilic granules, evidently derived from the fixed tissues. They were not found in the marrow but only in the vicinity of the trabeculæ; in our experience they are seen in the rat when fed diets which are low in calcium. Everywhere in the trabeculæ were encountered evidences of bone destruction for the most part carried on by small perforating blood vessels resembling those which destroy the cartilage at the cartilage shaft border. Trabeculæ were found which were very well marked by holes or pits with irregular margins and often containing fragments of bone and bone corpuscles. Other trabeculæ have lost an entire side apparently by process of erosion. Evidently when the diet is deficient in calcium, the

organism keeps replenishing the calcium in the circulating blood. The histological changes in the bones mentioned were remarkably constant irrespective of the diets employed. The histological changes in the bones which have been mentioned seem, therefore, to have been dependent on the quantities and relations of the calcium and phosphorus present.

Different diets have different rickets-producing properties apart from their contents of calcium and phosphorus. For the production of rickets there must be growth, not growth in weight, but growth of the skeleton. We believe it probable that a certain amount of the fat-soluble organic factor in the diet favors the development of rickets and is essential for its development; it now seems probable that a certain amount of the fat-soluble factor in the diet is essential for the development of the rickets.

Certain diets low in calcium and having phosphorus in concentrations not far from the optimal, when fed to the rat under the experimental conditions already mentioned, produce rickets. When the phosphorus in the diet is increased to high concentrations, whether by the addition of inorganic or organic phosphorus, the pathological condition in the bones appears to recede from rickets. Diets low in calcium but high in phosphorus seem to be, therefore, less effective in the production of rickets than diets low in calcium in which the phosphorus concentration is not far removed from the optimal.

NEW METHOD FOR THE DETERMINATION OF URIC ACID AND EVIDENCE AS TO ITS DIMORPHISM.

By J. LUCIEN MORRIS and A. GARRARD MACLEOD.

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.)

During critical investigation of colorimetric methods for the determination of uric acid certain conclusions were reached concerning the chemical principles underlying the reactions involved. The zinc precipitation of uric acid possesses many advantages over the silver precipitation. Sodium cyanide, in addition to its usefulness in forming double ions with silver or zinc, multiplies the amount of color obtainable from a given amount of uric acid. By use of this multiplying effect a definite maximum color is

attained when an excess of cyanide is present. When such a quantity of cyanide is used presence of sodium carbonate is undesirable. Cyanide gives a color with phosphotungstate in the absence of sodium carbonate. Among other conjugated tungstic acids, arsenotungstic acid was prepared and was found to obviate this difficulty. A new method, based upon these observations, was devised which possesses many advantages over former procedures.

Comparative results were secured from a large number of specimens of human blood by application of the new and the older methods. Some cases showed agreement but others showed marked irregularities. These irregularities were higher values obtained by the new method than by the old. All efforts to lower the new method results obtained in these cases failed. A means was found, however, by which the older method results could be increased so as to correspond with the new method results in those cases in which irregularities occurred, but the same means did not increase the values in the cases in which the two methods agreed. The nature of the modification of the old method used for this purpose suggested that the additional value was due to the presence of a second form of uric acid rather than any other substance. Further evidence to substantiate this conclusion is that uric acid was separated as such from large quantities of human blood in amount corresponding to the new method value.

A PHYSICOCHEMICAL METHOD OF CHARACTERIZING PROTEINS. III.

By EDWIN JOSEPH COHN.*

(*From the Laboratories of the Harvard Medical School, Boston.*)

In two earlier reports to this society we have pointed out: (1) that curves representing the fractional titration of proteins with acids and bases give a physicochemical method of characterizing proteins, and (2) that a comparison of the titration curves of a number of proteins showed differences in slope at their isoelectric points. This difference in slope was correlated with their solubility. It was shown that the slope of the titration curve of

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egg albumin (a protein that is soluble in water), was steeper at its isoelectric point than those of the slightly soluble globulins, and that the slope of glutenin (a protein insoluble in water but soluble in dilute acids and alkalies), was still less steep.

The titration of proteins, that are largely precipitated in the neighborhood of their isoelectric points, involves the heterogeneous equilibrium between precipitated and dissolved protein. This is being quantitatively studied in a number of cases by analysis of the concentration of total protein, of protein ions, and of hydrogen ions in the liquid phase.

It has been found:

1. that tuberin, euglobulin, pseudoglobulin, and casein have constant solubilities at their respective isoelectric points. The solubility of these proteins has been found to be independent of the amount of undissolved protein present, and approximately equal to:

	Per liter.
	gm.
Tuberin	0.4
Euglobulin	0.2
Pseudoglobulin	0.1
Casein	0.08

This solubility (S) must be considered to be made up of undissociated protein molecules, (HPOH), and of the dissociated protein ions, (PH^+) and (POH^-).

2. Upon the addition of an acid or an alkali to one of these proteins the solubility increases. In the case of casein it has been found that the increase in solubility is, within wide limits, proportional to the NaOH added. From this observation it follows that the amount of protein dissociated as base (HP^+) is negligible, and that the solubility of the undissociated molecules (HPOH), remains constant, as long as the heterogeneous equilibrium with the casein precipitate persists.

3. As long as the solubility of the undissociated protein is constant the mass law equation should assume the form:

$$(H^+) \times (BPOH) = K$$

where (BPOH) designates the soluble compound of protein and base, and K the solubility product constant. This equation has been found to hold for casein in the earliest stages of the reaction:

$$(\text{Casein}) + (\text{NaOH}) = (\text{Na caseinate})$$

where the protein may be considered as acting as though it were a monovalent acid. It varies, however, with variation in the protein concentration in a manner that is still being investigated.

The titration curve for casein has recently been determined by Loeb, and confirmed in this laboratory. This curve appears to have the hyperbolic form (characteristic of the solubility product) only for a short distance near the isoelectric point. Thereafter the curve assumes the S-shape characteristic of homogeneous buffer action. If the curve describing the solubility product is extrapolated, on the basis of the constant calculated in the early stages of the reaction, the amount of combination with base due to this group of valences may be subtracted isohydrically from the titration curve. It will then be found that a second curve is obtained still more characteristically S-shaped. By this graphical method casein may be seen to dissociate as an acid of at least two different strengths.

METABOLISM OF CALCIUM AND PHOSPHORIC ACID ON ISORACHITIC DIETS.

By J. F. McCLENDON.

(From the Department of Physiology, University of Minnesota, Minneapolis.)

In young white rats the skeleton contains 96 per cent of the Ca and 82 per cent of the P of the body. The P:Ca ratio is 0.5 in the skeleton and 0.6 in the body. Normal rats of from 22 to 45 gm. on a normal diet retained 10 to 18 mg. of Ca per rat per day and 7 to 9 mg. of P. The same rats at a weight of 76 to 110 gm. retained 20 to 37 mg. of Ca and 11 to 14 mg. of P. This diet contained 790 mg. of Ca and 570 mg. of P. When the P is reduced Sherman has shown that rickets develops. The following diets are divided into isorachitic groups. Group 1 produces definite rickets under the conditions of little exercise and light. Group 2 was doubtful because of disease of the animals. Group 3 causes

very slow growth of the bones and Group 4 more rapid growth. Rachitic rats suffer from respiratory insufficiency due to small size of chest due to soft ribs.

Group 1.

Diet.	Flour.	Casein.	Spinach.	NaCl	"Lime."	Yeast.	Wheat germ Ca per 100 gm.	P per 100 gm.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*
57	85	10	1	2	2		931	148
59	93	0	1	2	2	2	894	138
60	90	0	6	2	2		971	125
61	91	2	1	2	2	2	905	132

Group 2. Diet 58 = diet 57 + 100 mg. of cod liver oil per rat per day.

Group 3. Diets 57 to 61 + 0.2 per cent flour substituted by KH_2PO_4.

Diet.	Ca	P
62	962	222
64	900	171
65	970	173
66	923	181

Group 4. Diet 63 = diet 62 + 100 mg. of cod liver oil per rat per day.

Litter 12. Body weight 28 to 58 gm. 1 month old when placed on diets.

Rat No.	Sex.	Diet.	P balance P. D.	Ca balance P. D.	
			mg.	*mg.*	
I	♀	57	−1	+2	Group 1.
III	♂	59	−1	+1	
IV	♀	60	−2	+1	
V	♀	61	−1	+4	
II	♀	58	−3 (Pneumonia).	+2	Group 2.
VI	♂	62	+2	+2	Group 3.
VIII	♀	64	+1	+5	
IX	♂	65	+1	+4	
X	♀	66	+1	+4	
VII	♂	63	+5	+12	Group 4.

THERMAL EFFECTS ACCOMPANYING ALTERATION OF THE O_2 AND CO_2 CONTENT OF BLOOD.

By HOWARD W. HAGGARD.

(*From the Department of Applied Physiology, Yale University, New Haven.*)

Experiments to be reported demonstrate that the reaction of reduced whole blood with O_2 or CO is practically athermic. The combination of laked blood or hemoglobin solutions with either gas is exothermic. The amount of heat actually liberated is influenced by the state of aggregation of the hemoglobin. Results to be reported suggest the probability that there is an endothermic absorption which neutralizes the liberation of heat by the combination of hemoglobin with O_2 or CO. The shift of the equilibrium $HbO_2 + CO \leftrightarrows HbCO + O_2$ under variation of temperature depends upon the relative heating effects of the combination of the iron of hemoglobin with O_2 and CO. Absorption of CO_2 by the blood is attended with the evolution of heat, and the loss of CO_2 by the absorption of heat.

CHANGES IN THE COMPOSITION OF THE IRISH POTATO TUBER DURING GROWTH WITH PARTICULAR REFERENCE TO THE INFLUENCE OF COPPER SPRAYS.

By F. C. COOK.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Three early varieties of potatoes, Irish Cobbler, Early Rose, and Early Ohio, and one late variety, the Green Mountain, were grown in northern Maine during the 1921 season. Some of the plants were sprayed with copper sprays and others received no copper sprays. Analyses of the tubers made during the growing season showed a gradual increase of solids, starch, and nitrogenous constituents. The principal constituent of the tuber is starch, where the chief increase takes place during tuber growth. The increase of nitrogenous constituents was pronounced in the diamino and other base nitrogen group, although the soluble and the coagulable nitrogen groups increased.

The increase of solids, starch, and nitrogenous constituents was greater in the copper-sprayed than in the non-copper-sprayed

tubers. The copper-sprayed tubers gave a higher average percentage of ash than the non-copper-sprayed tubers. A decrease of reducing sugar, sucrose, insoluble nitrogen, and insoluble ash was observed as the tubers developed.

The copper sprays showed their favorable effect at the time the first analyses were made, when the tubers were just large enough for analyses, being about 1 inch in diameter. The three early varieties of tubers showed a higher percentage of sugars (reducing sugars plus sucrose) than the Green Mountain tubers, a late variety, at the time of the first and second analyses. It appears that a correlation may exist between the high sugar content in the early stages of tuber development and the rapid growth which the early varieties make.

The copper sprays not only usually give an increased yield of tubers but yield tubers with higher food value; *i.e.*, with more solids which means more starch as the potato is a starch-producing plant. The nitrogenous constituents of the tuber are also increased by the application of copper sprays. These changes were found in 1921 when no *Phytophthora infestans* was present in northern Maine. The influence of the barium-copper spray on the potato appears to be particularly favorable. Data are presented in detail showing for the first time the changes which take place in tubers during their development and the favorable influence of copper sprays on them.

PEPSIN AND TRYPSIN OF TISSUES.

By H. C. BRADLEY.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

The examination of tissues for pepsin and trypsin, as enzymes of the autolytic complex (Dernby) fails to show evidence of either one. Crucial experiments were carried on in which only one or the other of these enzymes could function as a catalyst of protein hydrolysis and in which they were compared to similar digests to which pepsin or trypsin was added. The results confirm the older findings and definitely disprove the presence of these enzymes.

HYDROGEN ION CONCENTRATION IN AUTOLYSIS.

By A. KOEHLER, E. SEVERINGHAUS, and H. C. BRADLEY.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

Measurement of the H ion concentration in autolyzing liver shows that at death an H ion concentration of about pH 7+ exists. Acidity develops for 24 hours; reaching a pH of about 6.4 or 6.5. For 10 days this H ion concentration shows a slight progressive diminution to about 6.7 or 6.8. Thereafter there is a very slow but definite increase of H ion concentration, which may at the end of 20 days reach 6.4 or 6.5 again.

If alkali is added to the digest, sufficient to practically stop autolysis (say pH 9.0) there is a rapid increase of H ion during the first 24 hours (to 7.8 in this case) and thereafter a slow progressive increase, so that in 20 days the reaction may approximate 7.0 very closely. The rapid increase of H ion is not accompanied by autolysis nor by sufficient CO_2 production during the first few days to account for the change in reaction. Oxygen absorption during this period is large, however, suggesting the formation of lactates or similar intermediate acid products which neutralize the alkalinity.

Where acid is added sufficient to produce the optimum autolysis (H ion about 4.5 to 5.5) there is much less striking change of reaction. The increased buffer effect of rapid cleavage to amino-acids does not greatly alter the pH. A digest starting at 5.3 reached 5.7 in 4 days and 6.0 in 20 days, showing a gradual tendency of the reaction to approach that of control.

THE RELATIVE VALUE OF THE SOURCES OF BASE FOR THE FORMATION OF SERUM BICARBONATE.

By EDWARD A. DOISY and EMILY P. EATON.

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.)

Defibrinated blood which had been equilibrated with 18, 40, and 75 mm. of CO_2 was centrifuged under oil. Serum chloride and bicarbonate were determined and corrections were applied for the change in volume of the corpuscles. The loss of chloride accounted for 65 to 75 per cent of the increase of bicarbonate.

$$pH_{38^\circ} = \text{colorimetric pH to} + 0.01\ t^\circ - 0.38$$

The determination is carried out as follows: Blood taken under mineral oil, either oxalated or not, is centrifuged in a stoppered tube. The plasma is removed and always kept under oil. Two 20 cc. portions of neutral 0.9 per cent NaCl are placed in clear glass 20 mm. tubes. 7 drops of a 0.04 per cent phenol red solution are added. The saline solution is then covered with oil. With a 1 cc. pipette, graduated to deliver between two marks, 1 cc. portions of plasma are transferred to the two saline tubes. The mixtures are stirred and the color is compared in a comparator block with that of the Sörensen M/15 phosphate standards containing phenol red. These standards are made up at 0.05 pH intervals and the plasma color is read to 0.01 pH. The determinations are accurate to within the combined possible error of the colorimetric and electrometric determinations; *i.e.*, (0.02 + 0.02) = 0.04 pH.

This method may be applied to other protein-CO_2-containing solutions, such as joint fluids, etc.; but in such cases the protein correction must be redetermined.

A METHOD FOR THE DETERMINATION OF THE TITRATABLE ALKALINITY OF BLOOD.

By ISIDOR GREENWALD.

(*From the Harriman Laboratory, Roosevelt Hospital, New York.*)

The blood is laked, the proteins are precipitated with picric acid, and the mixture is diluted to ten times the volume of blood taken. An aliquot of the filtrate is titrated with 0.01 N NaOH, using methyl red, and neutral red, phenol red, or cresol red, and, finally, thymolphthalein. The total picric acid is precipitated with nitron, filtered, and weighed. The weight is converted into its equivalent volume of 0.01 N NaOH by multiplying by factors obtained by the titration of known amounts of picric acid. The titration of the free acid in the sample is subtracted from this. The difference represents the alkali contributed by the blood to neutralize the picric acid to the reaction shown by the indicator. The values obtained are unaffected by the presence of small amounts of oxalate, by the exact amount of picric acid used, by the degree of saturation of the blood with oxygen or carbon dioxide

and do not change rapidly as the blood is allowed to stand. The error is less than ± 2 per cent if the equivalent of 3 cc. of blood be used and is not more than ± 4 per cent if only the equivalent of 1 cc. be used. Normal blood yields the equivalent of from 40 to 47 cc. of N alkali per liter to methyl red and from 29 to 34 cc. to thymolphthalein.

A METHOD FOR THE INVESTIGATION OF TOTAL BASE EXCRETION.

By CYRUS H. FISKE.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

A method, sufficiently rapid and calling for small enough amounts of urine to meet the requirements of short period metabolism experiments, has been devised for the determination of the sum of all the non-volatile strong bases in urine (sodium, potassium, calcium, and magnesium). It is based partly on well known and long used devices for the separation of these bases from other constituents (ashing with sulfuric and nitric acids, removal of phosphate with ferric chloride, and of the excess iron as the basic acetate) with certain refinements necessitated by the small scale of the method. The bases are eventually obtained as sulfates, and the determination is made by a sulfate estimation with the benzidine method.

PHYTIN AS A SOURCE OF PHOSPHORUS IN THE PREVENTION OF RICKETS.

By WALTER H. EDDY, H. R. MULLER, and HATTIE L. HEFT.

(From the Department of Physiological Chemistry, Teachers College, Columbia University, and the Department of Pathology, New York Hospital, New York.)

The announcement by Sherman and Pappenheimer of two diets (84 and 85) which are respectively rachitic and antirachitic has suggested the desirability of determining whether the antirachitic effect obtained by the addition of 75 mg. of phosphorus in 100 gm. of diet in the form of K_2HPO_4 is paralleled by other forms of dietary phosphorus. Phosphorus occurs in foodstuffs as inorganic phosphorus, as phosphoprotein, as phospholipin, and as

phytin. In papers soon to be published Pappenheimer, Zucker, McCann, and Gutman will report findings in regard to phosphoprotein and lecithin. The present paper deals with preliminary work on the rôle of *phytin* in this connection.

Microscopic sections are shown obtained from studies of a single litter of rats which indicate that when an amount of phytin is fed that furnishes about 75 mg. of phosphorus in 100 gm. of the basal diet, *i.e.* Diet 85 with phytin substituted in place of K_2HPO_4, protection fails. Growth facts and food intake figures are supplied that show that there was little difference in the growth rate of the three series studied and that the food intakes during the 30 day period were practically identical, 9+ gm. daily.

Preliminary results are reported on a much larger series of rats which at the time of preparation of this paper had been distributed over five series of diets as follows: Group I on Diet 84; Group II on Diet 85; Groups III, IV, and V on a diet which was modified by replacing the inorganic phosphate of Diet 85 by varying amounts of phytin, so proportioned as to provide 70, 105, and 140 mg. of phosphorus to each 100 gm. of the diet, respectively.

Figures were given for the inorganic blood phosphorus, as determined by the Bell and Doisy method, after 14 days on this diet. These figures seem to support the conclusions of Hess and Gutman in regard to the diagnostic value of this test as an indicator of rachitic conditions, if we consider solely the two control series. In the case of the phytin-fed rats, however, the figures obtained were extremely variable and failed to support the x-ray evidence. The x-ray evidence while confirming the efficiency and inefficiency of Diets 85 and 84 respectively, shows absence of protection in any of the phytin series. These results seem to suggest that even though we give phytin in amounts sufficient to double the amount of phosphorus supplied by the inorganic phosphate of control Diet 85 it fails to protect.

Previous experience has shown that final judgment should be reserved until microscopic sections are obtained and the above findings are reported as subject to revision in the light of such evidence at the end of the normal period.

THE INFLUENCE OF DIET UPON THE CONCENTRATION OF PHOSPHORUS AND CALCIUM IN THE SERUM OF RATS.

By BENJAMIN KRAMER and JOHN HOWLAND.

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

A study of the calcium and the inorganic phosphorus in the blood serum of rats has been made to determine how the concentration of these substances is affected by diets, especially those producing rickets. We have previously shown that in human rickets the concentration of inorganic phosphorus is low and that it increases during spontaneous cure or as the result of the administration of cod liver oil. The concentration of calcium is essentially unchanged. In rats upon a diet deficient in phosphorus and in the organic factor contained in fats, especially cod liver oil (a diet which regularly produces rickets in the rat) the concentration of inorganic phosphorus is much diminished. The calcium is unchanged as long as a sufficient quantity of this is contained in the diet. When the diet is deficient both in calcium and in the organic factor, the calcium concentration of the serum is lowered. Whether there is a phosphorus or a calcium deficiency of the diet with a lowered concentration of these substances in the serum, cod liver oil administration assists greatly in maintaining the normal concentration of these elements.

THE FATE OF CERTAIN SULFUR COMPOUNDS IN THE ANIMAL ORGANISM.

By CARL L. A. SCHMIDT and GUY W. CLARK.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

Experiments were carried out to determine the fate of certain sulfonic acid compounds, with especial reference to those concerned in bile metabolism. All substances were administered by mouth to dogs kept on constant diets. Taurine is excreted in the urine unchanged and not as taurocarbamic acid as claimed by Salkowski. Cysteic acid is deaminized but the remainder of the molecule is excreted in the urine unchanged. Administration of taurocholic acid does not lead to its appearance in the urine.

Isethionic acid is excreted in the urine, no oxidation of the sulfur to sulfate taking place. These experiments indicate that dogs can neither oxidize the sulfur in sulfonic acids to sulfates nor deaminize compounds in which the amino group is in the alpha position with respect to the sulfonic acid radical.

A NEW WEDGE COLORIMETER FOR THE COMPARISON OF SOLUTIONS CONTAINING TWO COLORS, AS IN THE COLORIMETRIC pH DETERMINATION.

A DEMONSTRATION.

By VICTOR C. MYERS.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.)

It has previously been pointed out[5] that with the use of two wedges in a modified Hellige colorimeter, it is possible to obtain all the shades of color in a given indicator from the acid to the alkaline side, when one wedge is filled with an acid solution of the dye and the other with an alkaline solution, both being made with buffer solutions of a definite pH. The utilization of the wedges of the Hellige colorimeter for this work has been under consideration for some time in this laboratory.

Barnett and Barnett[6] and Gillespie[7] have utilized essentially the same principle in the colorimetric measurement of the hydrogen ion concentration. The former authors employ a long narrow rectangular glass box having a diagonal glass partition, one being used for the acid and the other for the alkaline solution of the indicator, while the latter achieves the same result by having a small movable cup fitted over the plunger but inside the cup of a Duboscq type colorimeter.

The use of wedges which are individually movable provide a much more flexible system. The reading of the wedge containing the dominant color of the dye, *e.g.* the red in phenol red, characterizes the hydrogen ion concentration, the yellow wedge being employed simply to obtain a correct color match. This

[5] Myers, V. C., *Proc. Soc. Exp. Biol. and Chem.*, 1921–22, xix, 78.

[6] Barnett, G. D., and Barnett, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 127.

[7] Gillespie, L. J., *J. Bact.*, 1921, vi, 399.

being the case it may also be employed to correct for any slight error due to extraneous yellow pigment in the unknown.

Briefly, the colorimeter comprises a brass box 30 cm. in height, containing a rack and pinion arrangement for three wedges, the movement of the wedges being entirely within the closed box. Readings are taken from 100 mm. scales which emerge from the top of the instrument as the wedges are raised. The instrument is provided with prisms and an eye-piece in front and a milk glass plate in back for the entrance of light. For the latter a small lamp box may be substituted. A door at the side gives access to the wedges and to the cup for the unknown which is mounted on it.

With one wedge the instrument may be used as an ordinary colorimeter. The second wedge provides for bicolorimetric work, as in the pH determination. To obtain a perfect match of unknown solutions which are slightly turbid or colored a third wedge may be used.

A PRELIMINARY REPORT ON THE NEPHROPATHIC ACTION OF THE DICARBOXYLIC ACIDS AND THEIR DERIVATIVES.

By WILLIAM C. ROSE.

(From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston.)

Comparative studies have been made of the influence upon renal function of a number of dicarboxylic acids administered subcutaneously as their sodium salts. Rabbits were used as the experimental animals. After a single dose of 1.0 to 1.5 gm. of tartrate, the preformed creatinine may increase six- or eightfold, and the blood sugar and cholesterol double in quantity, in addition to enormous increases in non-protein and urea nitrogen as previously observed by others. On the contrary, blood chlorides do not increase, and usually manifest distinct decreases in amount. Since tartrate nephritis is generally believed to involve the renal tubules primarily, the behavior of the chlorides may afford additional evidence for their elimination by the glomeruli.

In contrast to the action of sodium tartrate, sodium malate is only slightly nephropathic, and the salts of the closely related succinic and malonic acids are entirely without influence upon

renal function. On the other hand, sodium glutarate (1 to 4 gm.) produces a marked retention of nitrogenous blood ingredients, accompanied by a decided fall in phenolsulfonephthalein elimination. The nephropathic action of sodium glutarate explains the observation of Baer and Blum that following its subcutaneous administration to phlorhizinized dogs, decreases occur in the excretion of urinary nitrogen, sugar, and acetone bodies.

The toxic action of sodium glutarate cannot be due to a precipitation of the calcium derivative in the renal tubules, as has been suggested in explanation of the effect of sodium tartrate, since calcium glutarate is quite soluble.

We are extending our observations to other dicarboxylic acids.

THE RELATION OF SPLENECTOMY TO GROWTH AND APPETITE IN THE RAT.

By ARTHUR H. SMITH and LEAH ASCHAM.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

An experimental study of the alleged effect of splenectomy on the growth and appetite in the white rat was made, using the standard feeding technique in vogue in this laboratory. Observations on seventeen rats, eight of which were the progeny of splenectomized parents, gave no evidence of an increase in appetite or a variation from the normal rate of growth.

A study of the erythrocyte count on five splenectomized rats which were the progeny of splenectomized parents showed no anemia following the removal of the spleen.

CREATINURIA AND GROWTH IN THE DOG.

By VICTOR JOHN HARDING and OLIVER H. GAEBLER.

(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)

In studying the effect of diets varying composition upon the creatine excretion of young dogs, the authors have paid particular attention to the concomitant effect upon growth. Growth has been judged by alterations in body weight and in nitrogen balance. The change from a high protein diet to a low protein

diet had previously been shown to be followed usually by a decrease in creatine excretion, though if the low protein diet were continued long enough a rise in creatine ensued. The change from a low protein diet to a high protein diet, however, is not invariably followed by a rise in creatine excretion. The creatine excretion may rise, remain constant, or fall, depending very greatly upon the growth change induced by the alteration in diet.

Changes in growth, following the introduction of an essential amino-acid into a diet in which it was probably the growth-limiting factor, are accompanied by alterations in creatine excretion. Similarly, the introduction of vitamines into a diet, in which they had previously been deficient, by causing rapid growth changes will bring about changes in creatine excretion.

THE INFLUENCE OF FASTING AND OF LOSS IN BODY WEIGHT ON THE CARBOHYDRATE TOLERANCE.

By A. I. RINGER.

(From the Montefiore Home and Hospital, New York.)

In this investigation, the carbohydrate tolerance of diabetic patients was studied. The plan was to determine the tolerance when the patient presented himself and to repeat these tests at intervals of years, so as to find out in what way the progression of the disease and the loss in body weight might affect it.

The patient's blood sugar was determined in the morning before breakfast. After that he was given 50 gm. of bread with one cup of weak tea. The blood sugar was again determined after the first, second, and third hours. The urine was also examined for the presence of glucose.

The same test was repeated after 1 day's fast. Invariably after 1 day's fast, the patient showed ability to utilize carbohydrate better than on the day before the fast.

These same tests were repeated on four patients who presented themselves after having undergone courses of treatment in which their body weights were reduced from 15 to 22 per cent and during which period they were kept on low caloric diets and in an aglucosuric state.

After such examinations it was found that the patients that had lost in body weight not only had no increase in their tolerance for carbohydrates, but had a decided diminution in their power to utilize carbohydrates.

AN ISOLATION OF PHENOL FROM BEEF BLOOD AND FROM HUMAN BLOOD.

By ALICE ROHDE DAVIS AND ELEANOR B. NEWTON.

(*From the Psychopathic Hospital, Boston.*)

Phenols have been isolated from normal urine and from blood in cases of phenol injection or phenol poisoning. We have found it possible to obtain phenol as a crystalline brominated compound from both normal human blood and beef blood using mercuric acetate and sodium acetate to obtain the difficultly soluble mercurated phenols. To a protein-free blood filtrate obtained by the use of heat and acetic acid followed by colloidal iron is added mercuric acetate in sufficient amount to make a 0.25 per cent solution and subsequently sodium acetate to make a 10 per cent solution. Phenol is partially precipitated together with mercury compounds of uric acid and of combined uric acid. On concentration a further yield of mercurated phenol occurs. These compounds may be decomposed with hydrogen sulfide in acid solution or by boiling potassium iodide solution. The free phenol is then distilled from acid solution. The distillate may have the odor of phenol and gives positive tests with ferric chloride, with Millon's reagent, and with bromine water. The precipitate of brominated phenol is recrystallized from alcohol and comes down in long needles which are colorless in small amounts and melt at 92° (uncorrected). The M. P. of tribromphenol is given as 91.5°.

ANTIKETOGENESIS.

THE KETOGENIC-ANTIKETOGENIC BALANCE IN MAN AND ITS SIGNIFICANCE IN DIABETES.

By P. A. SHAFFER.

(*From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.*)

A revision of the values[8] assigned to the ketogenic versus antiketogenic influence of protein, carbohydrate, and fat in the metabolic mixture, allows the approximate calculation of the

[8] Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 457.

amount of total hydroxybutyric acid produced by the catabolism of any given mixture of foodstuffs. (The main change in the values is made necessary by the fact that each molecule of glucose is ketolytic for *two* molecules of acetoacetic acid.)

The fact that the calculated expectation now approximately agrees with the actual excretion of acetone bodies in nearly all of about a dozen different subjects so far examined—normals, moderate and very severe diabetics—indicates that the underlying hypothesis is substantially correct, and that the evaluations of ketogenic versus antiketogenic influences are not greatly in error.

From these facts it follows that the *minimum* amount of food carbohydrate needed to provide a theoretical ketogenic balance can be approximately calculated for any given subject; and this figure is the absolute minimum of carbohydrate tolerance below which ketosis is *unavoidable*. The most useful formula expressing the relationship appears to be:

$$\frac{\left(\begin{array}{c}\text{Total calories of energy exchange}\\ \text{per 24 hours}\end{array}\right) - (100 \times \text{urine N})}{50} = \begin{array}{l}\text{Gm. food CH to provide}\\ \text{approximate ketogenic}\\ \text{balance.}\end{array}$$

If a diabetic has enough carbohydrate tolerance to remain "sugar-free" on a diet containing the calculated amount (or more) of food carbohydrate he will have little or no ketosis. The calculated amount allows no margin of safety against accidental increase in total metabolism from unusual activity or infection.

If the tolerance is lower the *only* recourse is to reduce the total metabolism (the amount of ketogenic material in the metabolic mixture) by rest, low protein diet, undernutrition, or fasting, until the (approximate) relationship expressed above is accomplished. For each gram of the calculated minimum of carbohydrate which is not burned (because of low tolerance or because not available in food) slightly more than a gram of total hydroxybutyric acid is excreted.

A COMPARISON OF ACETONURIA CAUSED BY DISEASE WITH THAT CAUSED BY DIETS LOW IN CARBOHYDRATE.

By ROGER S. HUBBARD, SAMUEL T. NICHOLSON, Jr., and FLOYD R. WRIGHT.

(From The Clifton Springs Sanitarium, Clifton Springs, N. Y.)

Mathematical ratios were proposed to express the molecular relationship between glucose and fat for any diet fed. For normal cases receiving enough food to maintain metabolic equilibrium the ratio was based on the foods ingested; for diabetics not in metabolic equilibrium the ratio was based on the foods burned in the body, as calculated from the basal metabolism, carbohydrate ingested, and sugar and nitrogen excreted. Charts were shown in which the values of these ratios were compared with the acetone excretion, and a similarity of response was shown in the two conditions. From a study of the values of the ratio which corresponded with a very slight increase of the acetone excretion above normal, it was concluded that the glycerol radical of the fats probably figures as a source of antiketogenic material to the extent to which it will yield glucose when fed.

STUDIES OF THE METABOLISM OF DIABETES.

By RUSSELL M. WILDER, WALTER M. BOOTHBY, and CAROL BEELER.

(From the Mayo Clinic and Foundation, University of Minnesota, Rochester.)

A chart is presented giving the more important data of feeding experiments in a case of diabetes of great severity. These experiments were designed to throw light on the rôle of ingested protein on the diabetic metabolism. Observations were continued for 11 weeks with daily determinations of the urinary excretion of nitrogen, sugar, ammonia, acetone bodies, total acids, and phosphates and frequent determinations of the carbon dioxide-combining power of the plasma, the sugar, acetone, and fat content of the blood and the respiratory metabolism.

The following conclusions are submitted:

1. The postabsorptive or *basal* metabolic level of the diabetic individual is materially affected by the previous diet. In the

undernourished patient it may be found as low as 32 per cent below the Du Bois standard normal for a healthy person of like age, sex, and surface. The ingestion of food containing 1 gm. of protein per kilo of body weight with fat and carbohydrate in such an amount that the daily maintenance energy requirements of the patient were exceeded, caused an elevation of the basal level. The ingestion of 3 gm. of protein per kilo of body weight per day caused a greater rise in the basal metabolic rate than occurred with isocaloric amounts of other foods. A cumulation of the specific dynamic action of protein seemed to account in the main for the elevation of the *basal* metabolic level which occurred.

2. The rate of sugar utilization of this diabetic individual was depressed by high calorie diets, being much more markedly depressed by protein than by isocaloric amounts of fat. This protein effect was not primarily due to the sugar and ketogenic substances, which the ingestion of protein throws upon the metabolism, but to some other more specific action of protein.

3. Throughout this series of experiments the rate of sugar utilization varied inversely with the basal metabolic level of the patient, rising as the basal metabolic rate fell, and *vice versa.* This behavior suggests a definite interrelationship of the two and the fact that ingested protein elevates the *basal* metabolic rate makes it seem possible that it is by this mechanism that it adversely affects the rate of sugar utilization.

4. Diets relatively high in fat but low in protein, and planned to contain nearly 2 gm. molecules of fatty acid to 1 of glucose on two occasions checked and controlled a dangerous and rising acidosis. The data of the metabolism of 2 days when acidosis was minimal indicated that on these days the actual ketogenic-antiketogenic ratio in the metabolizing mixture was 1.53 and 1.78 respectively. It is suggested that the proportion of fatty acid which will completely burn with a limited amount of metabolizing glucose is not the same at all basal metabolic levels, but may be increased by measures designed to depress the basal metabolic rate.

THE EFFECT OF LOSS OF CARBON DIOXIDE ON THE HYDROGEN ION CONCENTRATION OF URINE.

By E. K. MARSHALL, Jr.

(From the Department of Physiology, the Johns Hopkins University, Baltimore.)

In certain conditions the escape of carbon dioxide from urine may have a marked effect in lowering the hydrogen ion concentration. Concentrated acid urines as ordinarily obtained on a mixed diet do not change appreciably in hydrogen ion concentration when shaken with air although practically all of the carbon dioxide may be given off. When very dilute urines or neutral and alkaline ones obtained after administration of alkali are so treated, there is a distinct decrease in acidity or increase in alkalinity. Precautions similar to those used in the case of blood are necessary in determining the pH of these urines. Experiments on man and animals indicate that the lowest hydrogen ion concentrations reported for urine may be too low due to neglect of proper precautions to avoid the loss of carbon dioxide.

A STUDY OF THE METABOLISM AND THE RESPIRATORY EXCHANGE IN POULTRY DURING VITAMINE STARVATION.

By R. J. ANDERSON and W. L. KULP.

(From the Biochemical Laboratory, New York Agricultural Experiment Station, Geneva.)

In an endeavor to obtain some information regarding the changes which occur in the metabolism of an animal which is deprived of the water-soluble vitamine B, a series of experiments was made on poultry. This study included observations under normal conditions of diet and then a state of vitamine starvation was induced by feeding polished rice until polyneuritis was finally produced.

A diet of polished rice caused a loss of appetite in our experimental animals and the food consumption fell to a low level. The continued lack of vitamine B in the diet caused a serious impairment of the digestive functions which during polyneuritis resulted in a practical cessation of digestion and assimilation.

During vitamine starvation there was a decided fall in the intensity of the metabolism corresponding to the decreased food consumption. The basal heat production in some cases fell to more than 50 per cent under that of the normal basal metabolism and the respiratory quotients averaged from 0.73 to 0.80 about 18 hours after feeding. Respiratory quotients approaching unity were obtained, however, during the first 3 or 4 hours after feeding polished rice until a short time before active symptoms of polyneuritis developed.

But during polyneuritis, although the crop of the fowl contained much undigested rice, the respiratory quotient seldom rose above 0.75, indicating a nearly complete inability at that time to utilize this food.

HEAT ELIMINATION AND GASEOUS EXCHANGE IN GRAPEFRUIT DURING STORAGE.

By C. F. LANGWORTHY AND H. G. BAROTT.

(From the Office of Home Economics, States Relations Service, United States Department of Agriculture, Washington.)

The experiments here described belong to the same general series as those reported in 1919[9] for bananas and apples and in 1920 for celery and eggs, and like the earlier ones were carried out in the large respiration calorimeter of the Office of Home Economics. In the present case the tests, which were conducted December 2 to 10, 1920, were made with grapefruit such as are imported in large quantities from Porto Rico. Refrigerator ships cannot be obtained for this trade, the fruit is transported in the holds of ordinary vessels where there is no regular ventilation and the temperature averages from 75 to 80°F., and the loss through decay, molds, and similar causes is very great. The experiments were made at the request of and in cooperation with the Porto Rico Experiment Station in the hope that accurate observations of the heat elimination and gaseous exchange of the fruit under conditions comparable to those on shipboard might yield information of practical service to shippers.

123 kilos of commercial pack grapefruit were used. Single layers of these were placed in the calorimeter on shelves made of

[9] Langworthy, C. F., Milner, R. D., and Barott, H. G., *J. Biol. Chem.*, 1920, xli, p. lxix.

¼ inch galvanized mesh wire and set 1 foot apart. Differential thermocouples were distributed among the fruit so that its temperature could be determined relative to its surroundings and resistance thermometers made it possible to determine the temperature of both the air within the calorimeter and of the walls. When the fruit had been placed in the chamber, the latter was sealed, the circulation of gases started, and the temperature adjusted to 78°F., at which point it was kept throughout the 8 days of the experiment. Determinations of the gaseous exchange and heat elimination were made at approximately 24 hour intervals, each determination representing the mean value for the period just ended.

The heat eliminated varied from 1.7 to 2.6 calories per hour for the total weight of fruit. The carbon dioxide elimination varied from 1.8 to 2.4 gm. per hour. The amount of water vapor given off was also very uniform, varying from 23.8 to 25.6 gm. per hour. The oxygen absorbed showed more variation and ranged from 0.7 to 3.1 gm. per hour. This irregularity in the oxygen may be accounted for by the fact that the oxygen is determined by difference and not measured direct as is the carbon dioxide and that with such small quantities of oxygen, a slight error in the residual analysis due to the large amount of moisture eliminated might appear as a large percentage error in the oxygen figure.

Since the heat elimination even under these experimental conditions with excellent ventilation and constant removal of heat, was sufficient to keep the temperature of the fruit above that of the surrounding air, it seems reasonable to suppose that where there is no ventilation, as in the hold of many ships carrying grapefruit, the temperature of the fruit would ultimately become several degrees higher than when it was placed on board.

A METHOD FOR THE DETERMINATION OF LACTIC ACID IN BLOOD.

By E. L. Scott and F. B. Flinn.

(From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York.)

The method which we describe is based upon that described by Ryffel. We have so modified the method that the filtrate obtained in Folin and Wu's system of blood analysis is used.

We have obtained satisfactory results in using the filtrate from 5 to 20 cc. of blood.

An aliquot of the filtrate is evaporated to about 40 cc. in an evaporating dish upon the water bath or if less than this, it is placed directly in a 500 cc. distilling flask with side neck and enough water added to bring it up to 40 cc. It is then neutralized with solid Na_2CO_3 and 45 cc. of concentrated sulfuric acid are added drop by drop, the flask being held under running water to keep it from heating. The flask is then connected with a Liebig condenser and a steam generator and distilled with a current of steam for 30 minutes, or until 150 cc. of distillate have passed over. The receiver is immersed in ice water. In this distillation the temperature of the contents of the flask is raised rapidly to 155°C. and kept between 155 and 158°C. during the entire process.

The distillate is then neutralized with 2 per cent NaOH solution and redistilled into an Erlenmeyer flask which contains 20 cc. of a 6 per cent solution of KOH and 20 cc. of 0.04 N standard iodine solution. This flask is also immersed in ice water. When 100 cc. have distilled over, the flask is removed and slowly warmed to about 20°C. 20 cc. of 15 per cent HCl are then added and the contents titrated with 0.04 N standard thiosulfate solution.

If care is taken to prevent heating during the addition of the sulfuric acid and to keep the temperature during the first distillation within the range indicated, the results are very concordant.

The thiosulfate solution should be standardized by treating a known amount of lactic acid in exactly the same manner as that described for the Folin and Wu filtrate.

THE BLOOD SUGAR CONTENT OF CAPILLARY BLOOD AS COMPARED WITH THAT OF VENOUS BLOOD.

By ISAAC NEUWIRTH AND ISRAEL S. KLEINER.

(From the Department of Physiological Chemistry of the New York Homeopathic Medical College and Flower Hospital, New York.)

Twenty normal students were employed as subjects. Blood was obtained simultaneously from a finger and from a vein of the same arm and was analyzed by the micro method of one of the authors (K). In eighteen out of the twenty cases the results did

not differ by more than 0.01 per cent and in the other two by only 0.02 per cent. The average of all twenty is 0.136 per cent for the capillary and 0.130 per cent for the venous blood. The method was also compared with a macro method and the conclusion is reached that the analysis of capillary blood by this micro method gives results which, for clinical purposes, can be considered identical with the venous blood sugar.

THE CALCIUM AND MAGNESIUM CONTENT OF THE HUMAN FETUS.

By MAURICE H. GIVENS and ICIE G. MACY.

(From the Research Laboratories, Western Pennsylvania Hospital, Pittsburgh.)

Twenty-five fetuses, ranging from 90 to 400 mm. in length and consequently varying from 3 to 8 lunar months of age, have been dried and ashed. The ash has been analyzed for its content of calcium and magnesium.

The increase in length of the body appears to be at a gradual rate at all times. The total solids of the body increase gradually and slowly up to the fifth month, thereafter at a more rapid rate; likewise is this true of the ash and its calcium content. The magnesium figures do not become significant until about the seventh month.

Up to the third month the calcium requirement of the fetus approximates *in toto* 300 mg. of CaO and 60 mg. of MgO. From this time up to the eighth month, the demand for lime is much greater, relatively and absolutely. The magnesium content of the body varies considerably. At no time does it equal or even approximate the amount of calcium present in the body.

The total ash varies from 3.85 to 33.4 per cent of the dry body weight. Calcium oxide represents from 1 to 12 per cent of the dry body weight and from 24 to 50 per cent of the total ash. In the majority of cases MgO is less than 1 per cent of the dry body weight and from 3 to 15 per cent of the total ash.

For the first 3 or 4 months of pregnancy the fetal demand for CaO varies from 10 to 30 mg. per day, whereas for the total period of pregnancy it may average 100 mg. per day. Whether or not the total lime requirement of the fetus can be met from the mother's food intake remains to be investigated.

THE EFFECT OF CHANGES IN THE PROTEIN AND ENERGY OF THE DIET OF MILKING COWS UPON THE MILK YIELD AND UPON THE AMINO N OF THE BLOOD.

By C. A. CARY.

(From the Research Laboratories of the Dairy Division of the United States Department of Agriculture, Beltsville.)

In five experiments on cows the changes in the blood by which changes in either the protein or carbohydrate of the diet affect the yield and composition of milk were investigated. The animals were put on approximately adequate rations. In two experiments the energy was reduced 33 per cent; in one of these the protein also was cut 50 per cent and in the other it was unchanged. In two other experiments the protein was cut 50 per cent; in one the quality of the diet protein was also reduced and in the other it was kept constant. In the fifth experiment only the quality of the diet protein was varied. In the first four experiments the cows were finally put back on the original rations. The amino N of the blood and plasma and the yield and composition of the milk were followed. The results may be summed up as follows:

1. The cut in the amounts of energy or protein or both in the ration reduced the milk yield and the concentration of milk N. The concentration of lactose was unchanged, and that of milk fat was reduced only when the protein alone was reduced. The amino N of the blood and plasma was reduced except when the energy of the ration was unchanged and the quality of the diet protein was also reduced. Reducing the quality of the diet protein either with or without a reduction in quantity reduced the milk yield without reducing the amino N of the blood and plasma.

2. When these changes in diet were reversed the changes in the yield and composition of the milk were reversed, but these changes were not the exact reciprocals of those produced by lowering the rations. Only when the amount of protein was alone increased without any change of quality did the amino N of the blood rise. It was, in general, at first reduced.

The results indicate that changes in either the carbohydrate or protein of the diet affect the yield and composition of milk through

changes in the quality and quantity of the amino-acid mixture of the blood, and that the quality of the diet protein is an important factor in effecting these changes.

ACID-BASE METABOLISM IN INFANTS.

By ALFRED T. SHOHL.

(From the Department of Pediatrics, Yale University School of Medicine, New Haven.)

A new principle in the study of acid-base metabolism is presented. The food and excreta are analyzed for all the acids and all the bases. Their acid or base value is calculated in cc. of normal solutions. From the value of the intake is subtracted the value of the output. The remainder represents the balance. If the body is in equilibrium the balance is zero, for the output equals the intake. In acidosis, if base is excreted, it represents acid retention or abnormal acid production. If there is an acid excretion it represents a retention of base by the body. In infants of 1 year the retention is roughly 100 cc. 0.1 N base per day.

The acid-base value of the urine can also be determined by titration according to Palmer and Henderson. The acid excretion equals the free acid plus ammonia. However, if organic acid is present this must be determined and subtracted. In alkaline urines the carbonates must also be determined and subtracted. For as the organic acids bind base so the carbonates too unite with base and, therefore, represent base excretion. The carbonates, therefore, act as buffers and preserve neutrality in alkaline urines. Unless these corrections are made the acid-base value of the urine cannot be determined by titration.

A method is presented for the titration of the acid-base value of the stools. The principle is to determine the organic acid by extraction. The stool is then titrated in alcoholic solution and the acid-base value determined by subtraction. If carbonates are present they must be subtracted as in the case of the urine. In the stools the buffer value of the phosphates is unimportant. The neutrality is preserved by the fatty acids and soaps and by the carbonates.

RESPIRATORY QUOTIENT STUDIES IN SCURVY AND BERI-BERI.

By H. J. GERSTENBERGER AND C. W. BURHANS.

(From the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland.)

This study, begun during the close of 1916, was started with the idea of bringing evidence for or against the theory that scurvy was the result of a disturbed carbohydrate metabolism, caused, owing to the absence of or the reduction in the amount of antiscorbutic vitamine in the diet, by the establishment of a disproportion between the quantity of the antiscorbutic vitamine in the food on the one hand and its carbohydrate content on the other. The relative scarcity of scorbutic infants and early difficulties in getting scorbutic guinea pigs to take adequate amounts of food at the desired time was responsible for the inclusion of beri-beri in this study, a disease which according to Funk can be produced with greater rapidity by increasing the carbohydrate intake of the pigeon.

Whenever the opportunity was offered scorbutic infants were studied. Finally, by using Kellers malt soup, it was possible to establish satisfactory experimental conditions regarding scurvy and food intake in guinea pigs and to study the respiratory quotients in these animals.

The results of these studies bring evidence that scorbutic infants and guinea pigs, as well as polyneuritic pigeons can burn carbohydrates completely, and that, therefore, the study of respiratory quotients of animals ill with scurvy or beri-beri brings no evidence in favor of the correctness of the above mentioned theories.

CHEMICAL BLOOD CHANGES IN PNEUMONIA.

By JOHN A. KILLIAN.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.)

These studies comprise determinations of the non-protein and urea nitrogen, uric acid, creatinine, sugar, chlorides, and carbon dioxide-combining power of the blood in cases of pneumonia of various types. In some instances, also, parallel estimations of

the total nitrogen and chlorides of the urine have been made. A significant rise in the undetermined nitrogen of the blood has been noted about the period of the resolution of the pneumonia. Consequent to this, an impairment of kidney function develops, resulting in an accumulation of the nitrogenous waste products in the blood. The rise in the non-protein nitrogen is followed by an increase first, in the uric acid; later, in the urea nitrogen; and finally, in the creatinine. In fatal cases a rise of the creatinine to more than 4 mg. per 100 cc. was noted. An attempt has been made to correlate the changes in the chloride concentration and the carbon dioxide-combining power of the blood throughout the course of the pneumonia with the clinical manifestations of the disease.

FURTHER STUDIES ON THE REACTION OF DYING TISSUES.

By WITHROW MORSE and R. GOLDBERG.

(From the Laboratory of Physiological Chemistry, West Virginia University, Morgantown.)

It has been reported by others and by the authors that as tissue dies, the low alkalinity passes to a relatively high acidity. Preliminary experiments showed that this reaction takes place with great rapidity, and Morse and van der Heyde have reported the results of a study of mammalian liver using the gas chain method for estimating acidity. In this study the concentration of hydrogen ions within a few moments after excision of the liver was found to be in the neighborhood of pH 4.6. Criticism had been directed against these results since there must be time given for equilibrium to be reached, and in order to rule out this difficulty we have repeated the experiments, with modified technique, using the Sörensen colorimetric method upon ice-cold alcoholic extracts, following somewhat the technique of Fletcher and Hopkins in their work on lactic acid in muscle. The same average results were obtained in this procedure as we obtained with the electrometric method. Attention was then turned to lactic acid as being responsible for the sudden development of acidity. A basal figure for lactic acid estimated as zinc lactate by the zinc oxide method was found to be 0.035 gm. per cent wet weight of tissue. This figure approaches that obtained by Fletcher

and Hopkins and more recently by Foster and Moyle for basal lactic acid in frog muscle. This concentration of lactic acid is adequate to explain the acid figures. Kidney tissue gives similar acid figures.

CALCIUM METABOLISM IN TETANY.

By FRANK P. UNDERHILL, WILDER TILESTON, AND L. JEAN BOGERT.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

When compared to normal individuals under the same experimental conditions a subject with tetany, presumably of gastrointestinal origin, showed a normal type of behavior to calcium intake except that there was evidence of a greater tendency to store calcium temporarily on a calcium-rich diet. On the other hand, on a calcium-poor diet this stored calcium is eliminated to a much greater extent than occurs in the normal subject. These facts may, perhaps, be interpreted to mean that the organism with tetany shows a greater need for calcium than the normal individual but that in tetany the regulation of calcium equilibrium is in an unstable condition.

INDOLETHYLAMINE IN THE URINE OF PELLAGRINS.

By M. X. SULLIVAN.

(From the Hygienic Laboratory, Washington.)

About 40 liters of mixed urine of pellagrins were treated with lead acetate and with basic lead acetate and the filtrate, freed from lead, was concentrated to 1,500 cc. The concentrated solution, made 5 per cent acid with H_2SO_4, was treated with phosphotungstic acid in 5 per cent H_2SO_4. In the filtrate from the resulting precipitate, indolethylamine was found. The solution, freed from phosphotungstic acid by baryta and from barium by dilute H_2SO_4, was concentrated *in vacuo* to 300 cc. and filtered. The filtrate was strongly acidified with concentrated HCl and filtered from a precipitate which formed. The filtrate, concentrated to 100 cc., was treated with 95 per cent alcohol and

filtered. The filtrate, freed from alcohol, was treated with picric acid. A dark red precipitate of long needles occurred which, on recrystallization from acetone, had a melting point of 242°C. From the picrate a chloride was obtained, melting at 244–245°. A small amount of free base was likewise obtained of which the melting point was not obtained. The free base and syrup, however, gave an intense violet-blue color with glyoxylic and sulfuric acids as did the hydrochloride, likewise. From the color, crystalline shape, solubility, and melting point of the picrate, the melting point and the Hopkins and Cole's reaction of the chloride, and the Hopkins and Cole's reaction of the free base, it is evident that the compound is indolethylamine.

THE CHICK AS AN EXPERIMENTAL ANIMAL IN VITAMINE STUDIES.

A PRELIMINARY REPORT.

By A. D. EMMETT and GAIL E. PEACOCK.

(From the Biological and Research Department, Parke, Davis and Company, Detroit.)

Four series of feeding trials were carried out using young White Leghorn chicks in comparison with rats and pigeons. It was found that in modifying the rations for the chicks by increasing the nitrogen, introducing some roughage, and supplying a liberal amount of charcoal, oyster shell, and grit, that the birds were seemingly well adapted to studies where either the vitamine A or B was absent.

The chicks were all fed for the first 2 weeks after hatching in the most approved practical manner so as to tide them over the initial critical period of growth. They were then placed in confinement and fed on the same ration for 7 to 10 days when they were given the various experimental diets. The chicks fed upon the normal control diet grew and gained at the usual rate and had the appearance of healthy fowl. Those fed upon the vitamine B minus diet soon showed definite signs of the deficiency—ceasing to grow, losing in weight, and developing other symptoms of malnutrition, culminating in polyneuritis in 10 to 12 days. Hydropericardium, hypertrophy of the adrenals and gall bladder, and atrophy of testes were found on autopsy. The

chicks fed the vitamine A minus ration continued to gain normally for a longer time than did those on a lack of the vitamine B. They showed a weakness of legs; poor condition of the feathers; fading or bleaching of the pigment in the legs, bill, and toes; anemia; edema about the eye, followed by an ophthalmic condition similating xerophthalmia; beading of the ribs; atrophy of the testes, and hypertrophy of the adrenals and gall bladder. The period of duration was from 14 to 21 days. In the case of the vitamine C, the requirements of the chick appear to be much less than for vitamines A and B.

The advantages in favor of the chicks are: ease of obtaining large numbers of animals; the minimum amount of attention and lessened cost compared with the breeding of the rat; the greater uniformity of the animals and hence lessened degree of variation; and a shortening of the time and duration of the feeding tests, due to the apparent increased sensitiveness of the chick to the lack of the vitamines.

The possibilities of the chick in such feeding trials appear to suggest that they are both promising and practical.

CALCIUM IN EGG-SHELL FORMATION.

By G. D. BUCKNER, J. H. MARTIN, W. C. PIERCE, and A. M. PETER.

(From the Kentucky Agricultural Experiment Station, Lexington.)

Six lots of 7 month old White Leghorn pullets were fed for 8 months, restricted as follows:

Lot 1.	Grains	+	tankage	+ no mineral material.					
“ 2.	“	+	“	+ granite grit, *ad libitum*.					
“ 3.	“	+	“	+	“	“	“	“	+ oyster shell, *ad libitum*.
“ 4.	“	+	“	+	“	“	“	“	+ limestone “ “
“ 5.	“	+	“	+ limestone, “ “					
“ 6.	“	+	“	+ rock phosphate, *ad libitum*.					

Tankage (12.5 per cent of total feed) containing 6.4 per cent P_2O_5 was fed in the mash. The grit used contained 2.42 per cent CaO soluble in strong HCl.

Results obtained from the analyses of the leg bones and the egg-shells and the number of eggs produced in each lot indicate

that the calcium carbonate can be utilized by the hen for the production of egg-shells and bones but that the calcium in tricalcium phosphate can only be utilized for the growth of bones and not for egg-shell production. Also that calcium starvation is not the determining factor in the production of shell-less eggs.

THE HEAT OF ENZYME REACTION.

A STUDY OF THE HEAT PRODUCED IN THE CATALASE REACTION.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

In a previous paper on the catalase reaction[10] as well as in the publications of earlier investigators the fact has been noted that the velocity constant of the reaction in the first few minutes diminishes very rapidly. It seemed probable that these changes are due to temperature alterations, and the experiments here reported were performed to test this hypothesis. The results demonstrate that the catalase reaction is exothermic, and that it is accompanied by a definite heat production.

The experiments were made with a preparation of liver catalase. The volume of the mixture and its reaction (pH = 7) were kept constant in all tests. Only the relative amounts of catalase and hydrogen peroxide varied. The reaction took place in a thermos bottle, and the rise in temperature was measured with a special thermometer. Experiments with boiled solutions of catalase showed that only a negligible heat production (0.02°C. per minute of shaking) occurs which must be used as a correction in the experiments with the unboiled catalase. Without discussing the weak points of the technique, the heat production in the catalase reaction is now an established fact, as is shown by the data recorded below. The curves of heat evolved differ in no essential respect from curves plotted from data of oxygen set free in the reaction. The maximum heat production occurs in the first 5 minutes; *i.e.*, during the period when the velocity

[10] Morgulis, S., *J. Biol. Chem.*, 1921, xlvii, 341.

constant changes most abruptly. It is, therefore, concluded that the abnormalities in the reaction velocity are directly associated with the thermal phenomena accompanying the catalase reaction. The heat production is evidently conditioned upon the

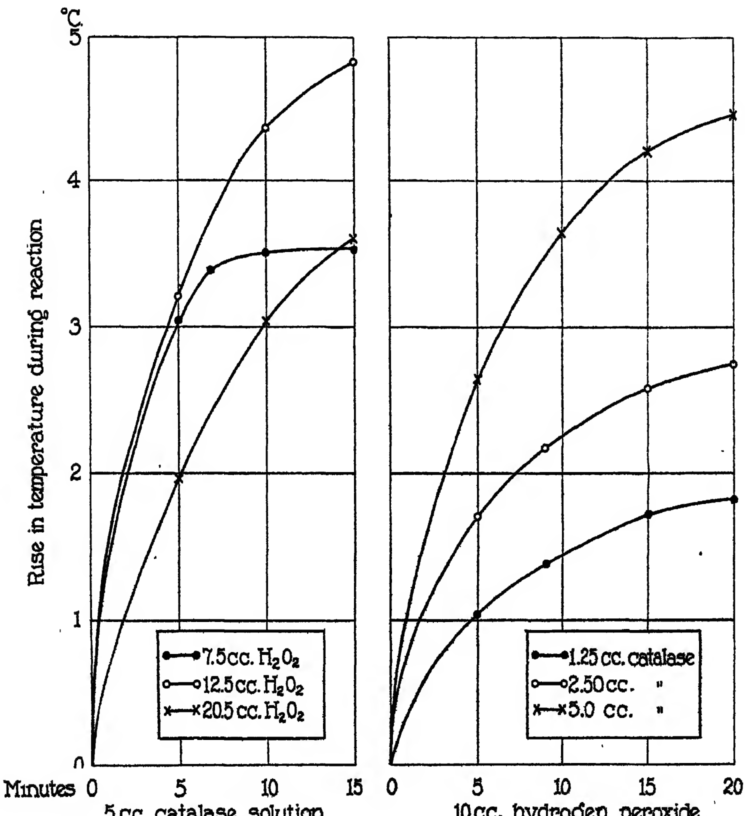

FIG. 1.

intensity of the reaction, and by further refinement of the apparatus it is expected that it will be possible to follow the progress of the reaction by the temperature changes not only for catalase but for other enzymes as well.

FURTHER EXPERIMENTS ON THE PREVENTION OF RICKETS IN RATS BY EXPOSURE TO LIGHT.

By ALFRED F. HESS.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.)

The development of rickets in rats fed the rachitic diet described by Sherman and Pappenheimer, can be prevented by daily exposures to direct sunlight for 15 minutes. Sunlight which has traversed flint window glass loses this potency. After having been reflected from a white surface it retains some of its effectiveness. Rickets, likewise, can be prevented by exposure for about 2 minutes to the rays from a mercury-vapor lamp. The ozone emanations from this lamp have no protective value. Rays from an open carbon arc lamp protect. Soft x-rays are ineffective.

Prolonged exposure to direct sunlight failed to prevent or to delay the onset of scurvy in guinea pigs.

GROWTH AND REPRODUCTION IN RATS ON A MILK DIET.

By H. A. MATTILL.

(From the Department of Physiology, University of Rochester, Rochester.)

The failure of adolescent growth and of reproductive ability in female rats on whole milk powder was not corrected by diluting the milk powder with lard, starch, and salts in varying proportions. The animals still showed the characteristic slowing of growth rate at adolescence and were infertile. Males grew normally but their reproductive efficiency was questionable. Additions of protein-free milk, of cod liver oil, or of traces of KI after the animals had reached the age of 150 to 175 days did not result in fertility or renewed growth. Ordinary stock rat food did not do it. When animals that grew up on stock food were transferred to milk rations at about the age of adolescence they likewise failed to rear their young, or were sterile.

The addition of a small amount of yeast to milk rations caused the females to cast litters regularly and repeatedly, but none ever lived more than a few days.

Satisfactory correlation between fertility and weight of gonads is not yet possible because the variability is too large for the number of animals thus far observed.

Of 16 males on milk rations	8 had average testes weights varying + 16 per cent from normal and
	8 had average testes weights varying − 42 per cent from normal.
Of 31 females on milk rations	6 had average ovary weights varying + 16 per cent from normal and
	25 had average ovary weights varying − 36 per cent from normal.

The plus figures are undoubtedly within the range of normal variability; the minus figures may not be. The partial success induced by yeast addition did not materially alter this distribution. Underdeveloped testes seldom, if ever, contained spermatozoa and histological material is now being studied.

RIGOR MORTIS.

By J. B. COLLIP.

(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)

When muscle plasma is carefully acidified, a precipitate of the proteins is brought about. This precipitate redissolves on the further addition of acid and reappears when alkali is slowly added, and again redissolves on the further addition of alkali. If one filters at the point of maximal precipitation the pH of the filtrate falls between 6.3 to 6.6 index. This corresponds to the pH index of muscle going into rigor. It is, therefore, suggested that the onset of rigor is due to physical changes, the development of post-mortem acidity in the tissue resulting in rigor when the isoelectric point of the proteins is reached. The passing of rigor may be associated both with the development of further acidity and with enzymatic action.

STUDIES OF THE NORTH AMERICAN SARRACENIACEÆ.

By JOSEPH S. HEPBURN, E. QUINTARD ST. JOHN, FRANK M. JONES, AND WILLIAM F. BAKER.

(From the Constantine Hering Laboratory, Hahnemann Medical College, Philadelphia.)

Liquor from closed pitchers of *Darlingtonia californica* contained diastase; protease, maltase, emulsin, invertase, and urease were absent. Liquor from closed pitchers of *Sarracenia flava* contained invertase and lipase; maltase, emulsin, diastase, urease, and esterase were absent; it had a surface tension of 66.4 dynes per cm. Protease occurred in the liquor from closed pitchers of the *Sarraceniæ*. Liquor from closed pitchers was bacterially sterile, that from open pitchers contained proteolytic bacteria. The pitchers absorbed nutrient compounds from their cavities. The pitcher liquor of the *Sarraceniæ* produced permanent cessation of motion and caused ants to sink more frequently and more promptly than in water. The liquor did not hemolyze human erythrocytes; that from closed pitchers had no toxic action when injected into the lymph sac of frogs or subcutaneously into a guinea pig. Prolonged administration of the mother tincture of either the rhizomes or the pitchers of *Sarracenia minor*, *Sarracenia flava*, *Sarracenia drummondii*, or *Sarracenia rubra* to rabbits by mouth in small doses produced allopecia, formation of red punctate umbilicated papules, rupture of the postules, formation of crusts, and other symptoms. The rabbits recovered when medication ceased, and had then acquired an immunity to the action of the drug. The rhizomes did not contain protease.

CHANGES IN THE REFRACTIVE INDEX OF THE BLOOD SERUM OF THE ALBINO RAT WITH TEMPERATURE.

By F. S. HAMMETT AND IDA S. TELLER.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

A study of the changes in the refractive index of the blood serum of the albino rat with rising temperature showed that two types can be differentiated according to the nature of the response. In the first type the changes in the refractive index coincide with

those of the solvent water and can be attributed to this serum constituent. In the second type, the curve of the changes of refraction with rising temperature falls away from that of water. This demonstrates a participation in the response of serum constituents other than that of the solvent water. The factors which contribute to this difference are unknown, although there is a possibility that a seasonal variation may be a determinant. It is certain that in this series the factors of body length, body weight, age, water content of serum both before and after the experiments, and previous state of digestion and absorption are not the causes of the difference between the two groups.

The correction for the reduction of the observed angle of refraction to the common base at 20° when readings are taken at different temperatures is obtained by the use of the formulas;

$$I = i - 1.25' \ (t-20) \text{ and}$$
$$I = i + 1.25' \ (20-t)$$

where I is the corrected angle of refraction; i, the observed angle of refraction; t, the observed temperature; and 1.25′, the change in minutes for each degree of change in temperature. These formulas hold for temperatures between 17.5 and 35°C.

TOTAL METABOLISM IN EXOPHTHALMIC GOITER.

By WALTER M. BOOTHBY and IRENE SANDIFORD.

(From the Section of Clinical Metabolism, Mayo Clinic and the Mayo Foundation, University of Minnesota, Rochester.)

The work included a quantitative study of the food intake, urinary elimination, the blood chemistry, and the respiratory metabolism in three cases of exophthalmic goiter. The total metabolism was found to be frequently in excess of 5,000 calories per day and occasionally over 6,000 calories, which is in marked contrast to the daily food ration of 1,500 to 1,800 calories common in many countries during the war.

THE METABOLISM OF INORGANIC SALTS.

By ERWIN G. GROSS and FRANK P. UNDERHILL.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

In normal dogs the relationships existing between calcium, magnesium, potassium, sodium, etc., in the blood are remarkably constant under fixed experimental conditions. When changes occur in one direction an immediate compensation occurs.

THE APPARENT ACID DISSOCIATION CONSTANTS OF OXYHEMOGLOBIN AND REDUCED HEMOGLOBIN.

By EDWARD A. DOISY, A. P. BRIGGS, and K. S. CHOUKE.

(From the Laboratories of Biological Chemistry, Washington University, School of Medicine, St. Louis.)

Determinations were made of the distribution of base between oxyhemoglobin or reduced hemoglobin and carbonic acid. From these data, the apparent dissociation constants of the two forms of hemoglobin were calculated.

The isoelectric points of oxyhemoglobin and reduced hemoglobin were determined by equilibrating the hemoglobin-base solutions with high tensions of carbon dioxide. The point at which the combined carbon dioxide equals the inorganic base of the solution was taken as the isoelectric point.

By plotting the bicarbonate values against pH, the relationship of loss of oxygen to increase of bicarbonate at a constant pH was obtained. The magnitude of these values was of the same order as those obtained on defibrinated blood. The loss of approximately 2 volumes of oxygen allows the blood to take up 1 volume of carbon dioxide without a change of hydrogen ion concentration.

COLORIMETRIC METHODS FOR THE DETERMINATION OF HOMOGENTISIC ACID AND MAGNESIUM.

By A. P. BRIGGS.

(From the Laboratories of Biological Chemistry, Washington University School of Medicine, St. Louis.)

Phosphomolybdic acid is especially susceptible to reduction by *p*-diphenols. Advantage of this reaction has been taken, first, to determine homogentisic acid in alcapton urine, and again to determine magnesium by the phosphorus content of the $MgNH_4PO_4$ precipitate.

ON THE RELATION OF THE HYDROGEN ION CONCENTRATION TO THE FERTILIZATION OF MARINE EGGS.

By G. H. A. CLOWES and HOMER W. SMITH.

(*From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.*)

The permeability of sea urchin and starfish eggs to homologous sperm varies with the hydrogen ion concentration of the sea water, regardless of whether the acid involved is carbonic acid or a mineral acid. In each species there is a hydrogen ion concentration at which the permeability to sperm is greatest, and another at which the permeability is zero. This indifference to the nature of the acid employed exhibits a striking contrast to the results on cell division reported in a preceding paper.[11]

ON PHYSICAL AND CHEMICAL VARIATIONS IN THE COMPARISON OF INTERIOR AND SURFACE PROTOPLASMA.

By G. H. A. CLOWES, ROBERT L. CHAMBERS, and IRVINE PAGE.

(*From the Biochemical Research Laboratories, Eli Lilly and Company, Indianapolis.*)

By comparing the relative cytolytic effects exerted by saponin and digitonin on the one hand, and hypotonic solutions on the other, it was possible to demonstrate that the surface of marine eggs contains a larger proportion of cholesterol than does the interior, or cholesterol is present in a more effective form as a protective agent against the action of saponin, digitonin, etc.

SOURCES OF ERROR IN THE DETERMINATION OF CHLORIDES IN BLOOD.

By ISIDOR GREENWALD.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

In the determination of chlorides in blood, it is usual to precipitate the protein by some acid precipitant. It is generally assumed that the precipitating anion completely displaces the chlorine ion from combination with protein. This is not always the case, particularly when nitric acid or metaphosphoric acid is used.

If picric acid is used as the precipitant, the presence of a purine, apparently hypoxanthine, in the cells makes the results too high

[11] Clowes, G. H. A., and Smith, H. W., *J. Biol. Chem.*, 1922, l, p. iv.

unless about 20 per cent nitric acid is present. The precipitate of hypoxanthine—silver-picrate—contains much more silver than the molecular formula given in the literature would require.

A MICRO METHOD FOR THE DETERMINATION OF UREA IN BLOOD.

By ISRAEL S. KLEINER.

(*From the Department of Physiological Chemistry of the New York Homeopathic Medical College and Flower Hospital, New York.*)

The method consists in direct Nesslerization of a Folin-Wu filtrate after the urea has been digested by urease. The color is compared, in the Klett-Kleiner micro colorimeter, with a 1 per cent potassium bichromate solution in a test-tube wedge mounted on a deep yellow glass background. The reading is made and the percentage of urea found by consulting a table.

ANALYSIS AND COMPOSITION OF CORN POLLEN.

By R. J. ANDERSON AND W. L. KULP.

(*From the Biochemical Laboratory, New York Agricultural Experiment Station, Geneva.*)

Having failed to find any reference in the extensive literature dealing with corn and corn products regarding the composition of corn pollen, we have undertaken an investigation of this substance. Since pollen plays an all important part in the process of fertilization and reproduction, it would seem as if some knowledge regarding the chemical compounds occurring in pollen would be of interest to plant physiologists.

Our results indicate that different varieties of corn produce pollen which varies greatly in composition and this fact might be of importance in cross-breeding.

The approximate composition of the pollen from three varieties of corn has been determined and a complete analysis of the ash of the pollen from one variety of corn has been made. Evidence is presented which indicates the presence of at least two phosphatides in corn pollen—one was an amorphous substance which also contained sulfur and the other was crystalline, corresponding in composition to a diaminomonophosphatide. Relatively large quantities of free inosite, *l*-proline, and choline were also isolated.

A COMPARATIVE STUDY OF THE COMPOSITION OF THE FEMUR.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

A few years ago, through the courtesy of Dr. S. J. Foote, I came into possession of an interesting collection of femurs. In view of the limited number of comparative determinations of bone composition, this material was examined chemically. Without entering here into a discussion of the analytical methods, it may be stated that the bones were cautiously freed from all traces of adhering tissue, dried, and ether extracted. The analyses were, therefore, made on water-free and fat-free material. The present report does not include the data for femurs of Egyptian Mummy or Pueblo Indian. The analyses extended to a determination of organic substance, ash, carbon dioxide, calcium, magnesium, and phosphorus. The quantitative data were calculated as calcium carbonate, magnesium phosphate, and calcium phosphate. Recognizing certain objections to this method of expressing the composition of the bone, it is, however, preferable to the conventional way of giving it in terms of percentages of Ca, Mg, P, and CO_2.

The composition of the bones examined reveals a remarkable uniformity. The percentage of CO_2 in the ash varies from 4.33 to 5.68, that of the Ca from 37.58 to 38.70, of Mg from 0.57 to 0.86, and that of P from 18.03 to 18.90 per cent. The femur of the turtle is an exception in that it contains a much greater proportion of CO_2 (8.43 per cent) and a lower percentage of phosphorus (17.02 per cent) than the other bones studied. Incidentally, it is also the softest bone with the largest content of organic matter.

Hoppe-Seyler regarded the salts of bone tissue as being similar to the mineral apatite $Ca_{10}\ Fl_2\ (PO_4)_6$ in which CO_3 takes the place of Fl_2. In a salt of this composition the Ca as phosphate and as carbonate should be in a ratio of 9.3:1 (930.87:100.08). The last column of the table gives these ratios as determined from our analytical data; the ratio will be seen to vary greatly, from 3.62 in the case of the turtle to 9.11 in the human femur. The ratios for the other femurs examined range all the way between these extremes. The lack of constancy in the ratio between the

calcium phosphate and calcium carbonate seems to invalidate Hoppe-Seyler's assumption.

Femur from:	Composition of bone.				Composition of ash.			
	Organic matter.	$CaCO_3$	$Mg_3(PO_4)_2$	$Ca_3(PO_4)_2$	$CaCO_3$	$Mg_3(PO_4)_2$	$Ca_3(PO_4)_2$	$\frac{Ca_3(PO_4)_2}{CaCO_3}$
Elk	29.40	7.20	1.59	62.50	10.19	2.55	88.50	8.68
Sheep	29.56	7.76	2.18	60.40	10.92	3.10	85.60	7.84
Mule	30.15	8.03	2.07	61.05	11.49	2.96	87.30	7.60
Hippopotamus	30.26	8.09	1.86	60.90	11.60	2.66	87.40	7.09
Turkey	30.51	7.48	1.86	61.45	10.76	2.67	88.30	8.42
Frog	32.36	8.45	1.66	58.35	12.50	2.45	86.30	6.89
Dog (bull)	32.98	7.42	1.58	57.50	11.08	2.35	85.80	7.98
Man	33.19	6.59	1.40	60.00	9.86	2.09	89.70	9.11
Horse	33.82	8.56	1.36	52.00	12.93	2.06	86.10	6.66
Turtle	37.23	12.03	1.95	49.85	19.16	3.10	79.40	3.62

A STUDY OF THE NON-PROTEIN CONSTITUENTS IN BLOOD OF SOME MARINE INVERTEBRATES.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

The non-protein components of blood have proved themselves an invaluable aid in the investigation of metabolism of the higher animals. This consideration led the author to undertake a survey of the blood of invertebrates. The present investigation is confined to arthropods from which sufficient blood for analysis can easily be obtained.[12]

The splendid system of blood analysis outlined by Folin and Wu has been followed in this research with some minor modifications necessitated by the nature of the material. This will be fully discussed in the detailed report. The blood from *Limulus*, blue crab, spider crab, and lobster was examined and the results tabulated below.

[12] It is a pleasure to acknowledge the help received from Dr. Leo Loeb in procuring the bloods.

Non-Protein Constituents of the Blood of Arthropods.

Animal.	Mg. per 100 cc. of blood.			Remarks.
	Sugar.	Non-protein nitrogen.	Uric acid.	
	26	13.0	2.0	
Lobster	20	12.8	2.5	
(*Homarus*).	19	12.5	2.1	
	20	13.3	2.4	
	45	33.0	1.0	Fresh batch.
	25	22.0	2.3	" "
Spider crab	27	23.0	2.3	" "
(Labinia).	29	19.5	2.3	" "
	26	16.0	3.8	?
	43	13.0	2.0	Several days old.
	43	15.0	1.0	" " "
	182	24.7	2.9	Fresh from the sea.
	64.5	22.7	3.4	" " " "
	41.5	8.0	0.3	Used on the day of delivery to laboratory.
	17.5	13.6	0.4	
Blue crab				
(Callinectes).	9.5	9.3	Negative.	1 day in aquarium.
	18.0	18.6	"	
	12.5	9.0	"	2 days in aquarium.
	13.8	10.0	"	
	34.0	26.0	0.3	Recently caught.
	22.5	20.0		1 day in tank.
Limulus.	12.5	14.0	0.7	2 days in tank.
	5.0	10.0		After many weeks in confinement.
	7.5	13.0	0.8	

The findings may be summed up as follows: the sugar, non-protein nitrogen, and uric acid content of the blood shows very great variability, except in the lobsters. The greatest variability occurs in bloods from blue crabs, the smallest in that of the lobster. The uric acid is present in large amount in both lobster and spider crab blood, in blue crabs only in specimens examined almost as soon as they have been removed from the water. The content of the non-protein materials of the blood evidently de-

pends on the condition of the animals (nutritive?). This is especially well shown in the blue crab which is an exceedingly active animal. Examined immediately upon being brought to the laboratory they showed a variable but high content of the non-protein substances, but after having been kept in the aquarium for 1 day the sugar and non-protein nitrogen diminish to practically the lowest level, while the uric acid has disappeared completely. The condition in *Limulus* is similar, and in the spider crab the same state of affairs exists though perhaps not quite so definitely expressed. On the contrary, the blood from lobsters shows very distinctly a stable composition of the non-protein components. The results suggest that in these arthropods different stages in the development of the mechanism for the regulation of the blood composition are represented. The influence of the nutritive state upon the blood composition, and its relation to the degree of development of the excretory mechanism will be the subject of direct experimentation.

THE REACTIVITY OF THE MOLYBDENUM AND TUNGSTEN REAGENTS OF FOLIN.

By VICTOR E. LEVINE and BERNARD C. BURNS.

(From the Biochemical Laboratory, School of Medicine, Creighton University, Omaha.)

To test out the reactivity of the Folin reagents 225 compounds of biochemical interest were employed. About 100 compounds responded to the phenol reagent of Folin and Denis. Among these were amino-acids, proteins, aldehydes, ketones, carbohydrates, and alkaloids. Creatinine was positive; creatine, negative. Most compounds reacted at room temperature while others required heating (formic acid, glucose, caffeine, etc.). In view of these findings the phenol reagent is worthless in the detection of the antineuritic vitamine, and the quantitative methods for phenol, cresol, and tyrosine must be modified so as to remove interfering substances from the compound under estimation. This criticism also applies to the estimation of adrenalin with the uric acid reagent.

The introduction of nitro groups in phenol renders the reagent

inactive. Mono-, *o*-, and dinitro- and trinitrophenol (picric acid) do not respond. Picraminic acid, however, is positive.

The uric acid reagent proved less reactive, only 45 compounds yielding positive results. Creatinine is positive; and creatine, negative. The estimation of uric acid does not suffer in accuracy from the non-specificity of the reagent, since in the method the uric acid is separated by precipitation from interfering substances.

In the system of blood analysis of Folin and Wu[13] there is recommended in the determination of sugar a reagent reacting with cuprous oxide. It is interesting to find that it reacts with inorganic reducers (cuprous oxide or chloride, ferrous sulfate, sulfites, sulfides, stannous chloride, etc.) and with only two organic compounds, which contain the hydrazine grouping—hydrazine and phenylhydrazine.

THE ACID-BASE EQUILIBRIUM IN THE BLOOD AFTER PARATHYROIDECTOMY.

By D. WRIGHT WILSON AND C. I. KRANTZ.

A METHOD FOR THE DETERMINATION OF URIC ACID.

By STANLEY R. BENEDICT.[14]

SOME PHYSIOLOGICAL AND CHEMICAL PROPERTIES OF THYROXIN.

By E. C. KENDALL.

SOME VARIATIONS NOTED IN THE ASH OF THE SWEAT.

By G. A. TALBERT.

NORMAL SUGAR EXCRETION IN RELATION TO CARBOHYDRATE INTAKE AND BLOOD SUGAR FLUCTUATIONS.[15]

By OTTO FOLIN AND HILDING BERGLUND.

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[13] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.
[14] Benedict, S. R., *J. Biol. Chem.*, 1922, li, in press.
[15] Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, in press.

PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND URINE ANALYSIS.

By W. S. McELLROY and H. O. POLLOCK.

A RAPID METHOD FOR THE DETERMINATION OF GLYCOGEN IN TISSUES.

By ELMER L. SEVERINGHAUS.

THE KETOLYTIC ACTION OF GLUCOSE IN VITRO.

By P. A. SHAFFER and T. E. FRIEDMANN.

A NEUTRAL GASTRIC ANTACID WHICH DOES NOT ACT AS A SYSTEMIC ALKALI.

By ISIDOR GREENWALD.

A STUDY OF THE BASAL METABOLISM, THE SUGAR TOLERANCE, AND THE GOETSCH TEST IN CASES OF THYROIDISM.

By JOSEPH S. HEPBURN and HARRY M. EBERHARD.

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